The Commission for the Investigation and Control of the
Chestnut Tree Blight Disease in Pennsylvania

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The Morphology and Life History

OF THE

Chestnut Blight Fungus

By PAUL J. ANDERSON, Field Pathologist

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The Morphology and Life History of the Chestnut Blight Fungus

By PAUL J. ANDERSON, Field Pathologist

Penn'a. Chestnut Tree Blight Commission

INTRODUCTION.

Considering that it has been only seven years since the first article on chestnut blight was published, the amount of literature on the subject is becoming extensive. Eighty-five of the principal contributions are given in the bibliography at the close of this bulletin, but none of these give us more than the briefest facts concerning the development and morphology of the producing organism, *Endothia parasitica* (Murr) And. To be sure, various authors have given such superficial facts as the size, shape, and color of the spores, asci and perithecia, the general times of years at which they occur, the macroscopic appearance of the stromata, spore horns and "fans;" the behavior of the organism in culture has been pretty well covered by Murrill (2, 3, 4), Pantanelli (34,89) and Clinton (83); inoculation experiments are recorded by Murrill (2, 3), Clinton (83), Rankin (101) and the writer (81). Interesting facts and observations have been added by many others but we know of no one who has made a detailed study of the life history and morphology. The necessity of this study is readily apparent; until such study is made we are dealing with an unknown enemy, our control measures are guess work and their success a matter of chance. The writer has not exhausted the subject by any means in the work which is recorded in the following pages. He presents the facts discovered with the hope that they may be of assistance to others who are working on this phase. The matter is presented under the heads of Spores, Mycelium, Pycnidia, Stromata and Perithecia, not because these all represent distinct stages and because they do not overlap, but because he finds it more convenient to group the facts about these heads.

The writer is under great obligations to Professors Whetzel and Reddick of Cornell University, Messrs. Detwiler, Carleton and Heald, officers of the Pennsylvania Chestnut Blight Commission, Messrs. Babcock, Kirk, Gates and Keefer, who have assisted him especially in the laboratory, and to a host of others who have sent specimens and given valuable aid and suggestions.

SPORES.

Like most other Ascomycetes, this fungus produces two kinds of spores: (1) pycnospores, otherwise known as conidia, conidiospores, asexual spores or summer spores and (2) ascospores, which are also called the winter spores or perfect or sexual spores. These will be treated below in the order named.

PYCNOSPORES.

On active young cankers during the spring, summer and autumn, slender, curling, yellow tendrils are especially abundant shortly after rain periods. If one of these "spore-horns" is put in water, it swells up and then apparently dissolves, but if a drop of this water is placed under the microscope, it will be found to contain millions of minute, hyaline bodies—the pycnospores.

Morphology. Murrill (4) who first described the species, gives their size as 1 x 2-3 microns, Clinton (92:367) as .75 x 2.5-4 microns, Pantanelli (89) as 1.7 x 3.8 microns. The writer made two hundred measurements of pycnospores from spore horns and got an average of 1.28 x 3.56 microns. An equal number of measurements was made of pycnospores produced in pure culture on oat agar and also of pycnospores from superficial pycnidia on wood, but the difference in size was found to be negligible. Their shape is shown in figure 52, being oblong of cylindrical with rounded ends, or slightly oval. As a rule they are straight, although occasionally slightly curved. Dr. Mickleborough's curved figures (19) are evidently exaggerated; they remind us more of the spores of a species of Naemospora which grows on the chestnut and the spore horns of which cannot always be distinguished macroscopically from those of *Endothia parasitica.* Although the tendrils of the latter species are bright yellow, the spores themselves, as seen under the microscope, are quite hyaline. This color is due to a pigment which is evenly diffused in the spore, or more likely the spore wall, and can be noticed only when there is a mass of them together. The pigment is the same as is found in the hyphae and will be discussed under the head of mycelium.

The wall of the resting spore is extremely thin and is not readily differentiated by staining. No markings, germ pores or layers can be detected. The spore is densely filled with protoplasm which is homogeneous; only occasionally are oil globules or vacuoles seen in the resting spore. By staining it can be determined that each spore contains a single small nucleus, which is elongated in the direction of the long axis of the spore. It usually lies close to the wall, about equi-distant from the ends, but may be almost in the end. It is shown

at the center of figure 14. With carbol fuchsin, and various other stains, a single body in each end of the spore stains very deeply. The significance of these polar bodies is uncertain. They cannot be located after germination and it is conceivable that they are used up in the enormous growth of the spore during that process. The outside of the spore is covered with a mucilaginous, sticky coat which is hard when dry and holds the spores together in the characteristic brittle "horns," but, on wetting with water, it first swells and then apparently dissolves and the spores float away free from each other. The mucilage of the spore horns is however, insoluble in alcohol.

Germination. Unlike the ascospores, the pycnospores do not germinate in cultures in water. Tap water, rain water, spring water and distilled water have been tried without success except that a slight and uncertain germination was secured in rain water. A small percentage of the spores germinated in water made slightly acid with sulphuric acid. A large number of media have been tried but mostly with disappointing results. Entirely successful germination was secured, however, in a decoction made by boiling chestnut bark in water, filtering and then sterilizing the filtrate in the autoclave. With this solution, a percentage of over eighty has been uniformly secured, and it has therefore been used almost exclusively in tests for longevity, vitality, etc. This suggests that there is some soluble substance in the bark of the chestnut tree that is necessary for their germination. In order to see if this substance is peculiar to the chestnut, sterilized twigs of the chestnut, red oak, white oak, black oak, sour gum, sumach, hickory, walnut, red maple and yellow poplar were sterilized in test tubes, and then washed with a suspension of pycnospores. From the fact that they germinated and produced the characteristic mycelium on all of these species, it is certain that the substance needed for germination is not peculiar to the chestnut tree, and that a spore would germinate just as readily if it fell into a wound of a sour gum or any of the other trees as it would on a chestnut. It is also significant that they will germinate perfectly in potato agar and most any of the ordinary nutrient agars. To determine whether they would germinate in the humus about the base of the trees if washed down into it by the rain, twelve petri dishes of sterilized humus were inoculated by spraying pycnospores over them. Not only did they germinate, but the mycelium grew and produced typical pycnidia on this medium. Tannin also is apparently not essential to germination because they germinate readily in media which are free from this substance.

Two methods of artificial germination have been used. In the first, a slide is supported on two glass rods in a petri dish as a moist chamber, and a drop of the bark decoction containing a suspension of the spores placed on the center of the slide. In the second method

a film of pycnospores in water is spread on a sterile cover glass and permitted to air dry. It is then covered with a drop of potato agar or some other nutrient agar and inverted over a Van Tieghem cell. This second method was used when it was desired to study the process of germination because it offered the advantages of keeping the spores stationary, and at the same time they could be put under the immersion lens.

The time required for germination varies widely with the temperature. Fulton (48:52) says: "Conidia germinate best at a temperature of 60 degrees F. and distinctly less rapidly at temperatures 10 degrees below or above that point." The writer, on the other hand, secured the most rapid germination at 89 degrees F., the shortest time secured for the appearance of germ tubes being twelve hours. At temperatures ranging from 60 to 75 degrees F., germination occurs in from 18 to 36 hours. At lower temperatures it often requires four or five days. No effort was made to find the exact maximum and minimum temperatures. Some experiments by D. C. Babcock in our laboratories indicate also that light hinders germination. From the data given, it appears that the very warm periods of the summer are most favorable for infection by pycnospores. That winter conditions are not favorable is indicated by the following experiment: At the beginning of every month during the last year, twenty-five or more inoculations in healthy chestnut trees have been made with conidia. At the present time, (June 15, 1913), none of those made after September or before April show any signs of producing cankers. Cankers are appearing about the inoculations made in April. Apparently then, infection will not necessarily result even if conidia do gain access to wounds during the winter.

The process of germination is preceded by an enormous swelling of the spores. This swelling begins in fifteen to twenty hours after they are placed in neat bouillon agar, and is then very rapid until the germ tube is pushed out. As previously stated, a mature spore measures about 1.28 x 3.56 microns. At the end of 18 hours, 50 spores which were just on the point of pushing out germ tubes, gave an average of 6.86 x 10.53 microns. The largest one observed was 9.05 x 14.48 microns. The volume of the spore just before germination is thus more than eighty-five times that of the resting spore. This increase in size is shown in figure 38, at the center of which are a number of resting spores. The various shapes assumed by the germinating spores will also be observed here. They may become cylindrical, oblong, elliptical, isodiametric, ovate, pyriform, reniform or dumb-bell shaped. in which latter case they resemble ascospores. The contents become coarsely granular, and often large vacuoles are seen, due to the rapid swelling. The first indication of a germ tube is a small protrusion or

pimple at one end which rapidly increases in length. So far as observed, the tubes are always at the ends of the spores. A few hours after the beginning of the first tube, another one starts at the other end of the spore. Only very rarely do both start at once. The rate of growth, size of the tubes and order of the laying down of the septa are brought out by the series of camera lucida drawings of single spores at short intervals reproduced in figures 39 and 40. This is an average growth in potato agar in Van Tieghem cells at 21-26 degrees C. The pycnospores generally produce two germ tubes. Very rarely a third one comes out laterally. From three to six hours after germination starts, the first septum appears in the tube and other septa are laid down often enough after that to make the cells of the mycelium 4-10 times as long as broad. As the germ tube lengthens, the cells composing it increase in diameter but the septa, being solid plates, do not increase in size correspondingly; hence the constrictions at the septa which become more marked as the mycelium becomes older (Fig. 8). Sometimes a septum divides the spore during this process. After a time it is difficult to locate the old spore since the first cells of the germ tube become exactly like it, and it is now merely one of the cells of the hypha. The branching of the germ tube is shown in the figure just referred to.

The swelling of the spores is due not merely to a mechanical imbibition of water; it is really a process of growth. To be sure, dead spores will swell, but only to about half the size acquired by living spores. Pycnospores, stained just before the germ tube is pushed out, show that the increase in size is accompanied by active nuclear division. Even at this time, two to six nuclei, rather larger than the original nucleus, may be made out. Also the polar bodies have disappeared and the protoplasm is not dense. The nuclei push out into the germ tube almost as soon as it starts. The wall in the meantime has increased in thickness until it almost equals the diameter of the resting spore and is quite distinct in stained sections. A germinating spore is shown in optical section in figure 13.

Vitality. All experiments up to the present indicate a remarkable vitality of the summer spores. Reasoning from analogy to what is known or believed of the imperfect spores of most fungi, one would not expect them to survive winter conditions. But the case is quite the contrary. During every month of the past winter pycnospores were taken from the woods, (a) from spore horns, (b) from pycnidia imbedded in the stromata and (c) from superficial pycnidia on bare wood and tested for germination in bark decoction. The percentage of spores which germinated ranged between 54 and 71 per cent., being only slightly lower than that of fresh conidia in culture, and showing only slight variation for the months. Apparently, then, weather conditions such as we have had in Pennsylvania during the

past winter, have very little if any effect on their vitality. Heald and Gardner (93) also found that freezing does not affect the vitality of the pycnospores. Tests made at various times during the summer of 1912 show also that the hot and dry weather of summer does not affect their vitality. Three series of tests were conducted to determine their longevity. In the first series, spore horns were detached from the bark and stored in open vials in the laboratory. At the end of each month, sterile twigs have been inoculated with the spore horns. Every test has been successful, including the last, which was at the end of one year. In the second series the spore horns were left attached to the bark, which was kept dry in the laboratory, and germination tests made in decoction as given above. The last test—at the end of 11 months and 15 days—gave a germination of 65 per cent. In the third series, pycnidia in the bark were stored. This series has been running only eight months; the last test gave a germination of 40 per cent. All these series are being continued and there is little doubt that they will retain their vitality much longer than a year since very little decrease in the percentage has been noticed. On the other hand, if the conidia are separated by dissolving the spore horn in water and then dried, they do not retain their vitality very long. The writer has not seen them germinate when kept in this condition longer than one month, but more experiments are necessary.

Inoculation experiments with conidia are described in detail by the writer and Babcock in Bulletin 3 of the Pennsylvania Chestnut Tree Blight Commission. In general it has been proved that almost any kind of a wound in the bark may be infected with pycnospores, whether they are introduced dry or suspended in water.

ASCOSPORES.

On older cankers, as shown in figure 46, the mature stromata are beset with projecting papillae. The black speck at the apex of each papilla is the opening of a little flask in which the winter spores are produced.

Morphology. The shape of the spores is shown in figure 37, being oblong to oval with rounded or more or less blunt pointed ends, 2-celled and constricted at the septum when mature. Clinton (92:368) in Connecticut, evidently does not consider the constriction as constant. His photomicrographs however—as they have been reproduced in his plate XXVIII—show beautifully constricted spores. They are quite hyaline both as seen under the microscope and when seen in mass. Murrill (4) gives their size as 9-10 x 4-5 microns, Pantanelli (89-73) the same as Murrill, Clinton (92:368), says they vary from 6-10 x 2.75-5 microns and average (92:427) 7.45 3.2 microns, based on the measurement of one hundred spores. His measurements

are the smallest of any we have seen. The average of one hundred and forty measurements made by H. W. Anderson and reported in Bulletin 4 of the Pennsylvania Chestnut Blight Commission, was 8.53 x 4.49 microns. These were from points in Pennsylvania. In the same bulletin seventy-five measurements of ascospores made by Rankin in New York are reported and give an average of 8.8 x 4.4 microns. One hundred measurements of spores from points in Pennsylvania and Maryland more recently made by the writer gave 8.68 x 4.51 microns as the average.

The walls are thicker than those of the pycnospores and are also more resistant to chemicals. With strong sulphuric acid they may be made to swell until their thickness often equals the diameter of the contents but they do not dissolve. This treatment shows no stratification of the walls and no germ pores or markings of any kind. The septum is also swollen greatly by this reagent; in fact, in none of its reactions does it seem to differ from the wall, and it is evidently of the same composition. It is a true septum and not merely a dividing line between the protoplasts. This fact was particularly noticed because Saccardo in his description of the genus Endothia intimates that it is a false septum, and also because it differs in this respect from the long-spored southern Endothia, as reported by H. W. Anderson before the American Phytopathological Society in January, 1913.

The spore is densely filled with homogeneous protoplasm. Only occasionally have anything like oil globules or vacuoles been seen. The writer has not found the large globules (or vacuoles), represented in Murrill's figures (4), to be common. Chemical tests have shown no glycogen or other storage products except proteids. As shown in figure 37, each cell of the spore contains two or four nuclei; occasionally there is one or three, and in some cases the number is not the same in both ends of the spore; more than four in one cell have not been found. The nuclei are best brought out by staining with iron-alum haematoxylin. The ascospores, like the pycnospores are sticky and adhere with great tenacity to any object with which they come in contact. The nature of the sticky covering has not been exactly determined, but it is conceivable that it is due to the matrix of epiplasm in which the spores lie while in the ascus.

Germination. They readily germinate in tap water, spring water, rain water or any of the ordinary media used for this purpose. A higher percentage was secured in chestnut bark decoction, however, than in pure water but as a rule more than ninety percent germinate even in water. They germinate as soon as mature without a period of rest. Spores were produced in September from inoculations made the previous June, and as soon as mature, were tested and gave a good

percentage of germination. The same methods for artificial germination were used as were described in treating of the pycnospores.

The time required is much shorter than for pycnospores. At room temperatures they push out a tube in from six to twelve hours. The shortest time secured was one hour and twenty-five minutes after ejection from the perithecium. As for the effect of temperature on germination, Fulton (48:52) says: "Ascospores germinate best at a temperature of about 70 degrees F., but a good percentage of germination occurs at 85 degrees and 45 degrees F. Even at 38 degrees F. the germination of ascospores was 25 per cent in 24 hours and reached 70 per cent in three days."

Like the pycnospores they swell before germination, but not to such an extent. The resting ascospore measures approximately 4.5 x 8.5 microns. Fifty spores measured after ten hours in nutrient agar averaged 7.27 x 13.84 microns—representing an increase of about four times the volume of the resting spore. The largest one was 17.2 x 9.05 microns. During the swelling the shape remains practically the same except that the sinus becomes deeper. The first germ tube usually appears at the end, but this is not always the case—sometimes it is lateral. The second tube to appear is in the other cell; this is generally followed by a second one from each of the cells, making a total of four germ tubes, which is the rule for the ascospores of this species. Their order of appearance, size, manner of septation and branching is best explained by reference to the successive camera lucida drawings of single spores in figures 41 and 42. The germ tubes from the ascospores grow much more vigorously than those from the pycnospores. By sowing ascospores on chestnut bark agar, in summer weather, mature pycnidia have been produced in five days. The early and rapid development of the mycelium from the ascospores is probably due to the larger amount of food material available in the spores.

During germination the contents of the spore becomes granular and vacuoles often appear. The nuclear behavior is the same as that of the conidia described above.

Vitality. So far as has been determined, weather conditions have no effect whatever, on the vitality of the ascospores. During every month for the last year they have been collected from the woods and tested, but the differences in the percentage of germination for the months have been entirely negligible. Their longevity is indicated by the following two series of experiments: In the first series, ascospores ejected from the perithecia were caught on glass slides and then stored and tested every two weeks for germination by covering them with a drop of water. They continued to germinate for five months and six days. After that they would not germinate. In the second series, bark containing mature perithecia was stored in the labora-

tory and tested every month. The last test—at the end of approximately twelve months—gave a germination of above 90 per cent. There is no doubt that this experiment will give a much longer record, since they germinate almost as well now as they did a year ago. These experiments also show that the spores will live much longer when they remain in the perithecium than if they are ejected and free from each other. These tests of course, indicate only the time they would retain their vitality if they were kept dry. If, on the other hand, they were in a moist place, they would germinate at once and unless they gained entrance to their proper host or possibly, some suitable substratum for a saprophytic existence, they would die without causing any damage.

The results of a large number of inoculation experiments are given by the writer and Babcock in Bulletin 3 of the Pennsylvania Chestnut Tree Blight Commission. In general, the same thing may be said of them as was said of the pycnospores; any kind of a wound in the bark deeper than the cork layer may be readily infected either by dry ascospores or with ascospores in suspension in water. In fact, there seems to be very little difference in the ability of the ascospores and pycnospores to produce the disease on the trees.

MYCELIUM.

This is the absorbing system of the fungus. It consists of millions of fine branching threads—the hyphae—which grow into the living tissues of the bark and sap wood, killing and digesting them in its progress round the tree. It is thus the immediate agent in producing the canker and ultimately killing the tree.

In culture. The beginning of the mycelium is the germ tube; the mature mycelium with its millions of hyphae is produced simply by the continued elongation and branching of the germ tube. In all essential points it is alike, whether produced from an ascospore or a pycnospore. A few hours after the germ tube starts it begins to divide into cells by laying down septa. (See figures 38-42.) Shortly afterwards, branches are pushed out from these cells and these in turn become septate and give off branches until a thick tangle of filaments is produced. These processes, so readily followed in the simple germ tube, are in all essentials the same in the later growth of the mycelium. Branching is nearly always preceded by septation; it is always monopodial and it is very rarely that more than one branch is produced from a single cell. The sinus at the septum, seen in the younger mycelium, is less distinct in older hyphae. The manner of branching is shown in figure 8. The individual hyphal cell is best studied in agar culture although it shows some slight differences from the cell in the bark, as will be explained later. The diameter of the hypha in agar culture varies from 2 to 12 microns, and the length of the cells

from 20 to 50 microns. The apical cells have very dense protoplasm, but, further back in the hyphae, large vacuoles appear, as shown in figure 8. The protoplasm is not homogeneous but shows larg granules and certain refractive bodies. The wall is very thin and easily collapses when dried. Each cell contains several small nuclei as shown in the figure.

The yellow pigment. The mycelium grows luxuriantly on a large number of artificial media. Cultural studies have been reported in detail by Murrill (2) and Clinton (83). Results secured by the writer largely duplicate theirs, and will not be recorded here. For ordinary purposes the writer has used potato agar. On this medium, at the end of from four to six days the mycelium begins to turn yellow, due to the production of a pigment in the cells. The same pigment gives the characteristic color to the spore horns and the stromata on the bark. It is apparently evenly diffused in the cells or cell walls. The writer has noticed that old agar cultures of the fungus often become purple or wine colored. Other experimenters have told him they have had the same experience and were at a loss to explain it. The connection between the purple color and the yellow pigment, as worked out by H. W. Anderson, is this: The pigment is yellow and insoluble when in an acid or neutral medium, but in an alkali medium is readily soluble and takes on a purple color. This can readily be demonstrated by pouring a solution of sodium hydroxide or any other alkali over the yellow mycelium. The fungus, in its growth on the agar, gradually causes it to become alkaline in character, and the pigment goes into solution and colors the medium purple. Pantanelli (34) says that the pigment is a lipochrome. Quite recently it was isolated and its chemical reactions determined in some detail by Cecil Thomas of Wabash College.* In this excellent piece of research, he shows that it does not resemble a lipochrome in any way except in color and solubility but that it is one of the colored compounds known chemically as the aurines. It is best isolated by extracting with alcohol and then precipitating with hydrochloric acid.

The fans. In order for the germ tube to gain access to the host tissue the spore must germinate in a wound. As reported in Bulletin 3 of the Pennsylvania Chestnut Tree Blight Commission, all attempts to produce infection without a wound have failed. The germ tube is not able to bore through the cork layer nor to enter through lenticels. Even if one secured an occasional infection without making a wound, it would be difficult to prove that the bark was free from small abrasions which had escaped the notice of the experimenter. But if germination takes place in fresh wounds, the germ tube will thrive on the injured and dead cells until it has produced a mass of mycelium. Then, gradually accumulating strength as it increases in size, the mycelium *en masse* pushes out through the living tissues of the bark.

*Master's Thesis. Publication of the Botanical Department of Wabash College.

Single threads do not seem to possess the power to penetrate alone among the living cells. Starting from a narrow point, the hyphae grow out in ray-like bundles, completely destroying the parenchyma and collenchyma and cambium cells as they go. All the rays starting from a single point are contiguous and they form a fan-like mat of mycelium as shown in figure 50. These fans are flat because they are not able to destroy the segmental bast zones but must squeeze between them. The edge of the fan is quite regular and is surrounded by a darker gelatinous band of the disintegrating host cells. Whether the cells are killed by a toxin secreted by the parasite or whether they are killed by the mechanical action of the mass of hyphae was not determined. The fans vary in length from one-eighth to three-quarters of an inch. The young ones, on the advancing edge, are pure white but as they become older they become light yellow or buff in color. This color, however, is not due to a development of pigment, since the pigment is never found in the fans; it is probably due to a decomposition product of the disintegrating host cells, which stains the mycelium. Each ray consists of a loose bundle of hyphae running almost parallel and branching only sparsely. They are much more uniform in diameter than the hyphae in agar culture. They are about 7 microns in diameter and are divided into cells about 30 microns long. They are not anastomosed in any way; a section of a ray showing their relation is represented in figure 9. The individual cells of the hyphae are densely filled with rather coarsely granular protoplasm. As the fans become older, however, the cells become vacuolate. Like most of the other cells of this fungus, they are multinucleate. The fans are produced only in the growing season. Although the canker spreads slowly in the winter, no white fresh fans are found in that season.

Rate of growth. The rate of growth of the mycelium under natural conditions on the tree can be measured by the increase in the size of the cankers. During the last twelve months, a large number of cankers have been outlined at the end of each month as shown in figure 49, and the averages computed for the months. Table I gives the increase in diameter during the last year. The increase in length—up and down the tree—is greater but not so important since it is not the growth in this direction that kills the tree. The table shows the effect of winter temperatures on the growth. The last winter in Pennsylvania, however, was exceptionally mild, especially the months of December and January.

Even the most rapid growth in the summer time—as indicated by the table—is less than one millimeter per day. But on artificial media, such as chestnut bark agar, the writer has often seen a growth of three millimeters per day. Also, in the dying bark after the tree is cut, the mycelium will spread at a much more rapid rate than when

it is invading the bark of a healthy tree. In the latter case, it does not advance by producing fans but by individual strands.

TABLE 1.

Showing the monthly rate of growth of cankers. Transverse diameters of the cankers.

Month.	Number of cankers.	Average growth per month in centimeters.
June, 1912,	31	1.88
July, 1912,	200	2.78
August, 1912,	186	2.83
September, 1912,	140	1.85
October, 1912,	53	1.92
November, 1912,	27	0.00
December, 1912,	27	*1.35
January, 1913,	89	.51
February, 1913,	89	0.0
March, 1913,	84	.7
April, 1913,	21	1.1
May, 1913,	41	2.4

*Doubtful record. No growth at all on a large number of other trees examined.

Vitality. The mycelium, like the spores has a remarkable vitality. That it is not injured in the least by low temperatures in winter is proved by the fact that successful isolations were made from under the bark during every month of the last winter, and also by the vigor with which the canker resumes growth in the spring. To see if freezing would affect it when exposed while growing under artificial conditions, colonies were started on agar plates which when they were about one inch in diameter, were put out of doors and kept frozen up during the whole month of February which was the coldest month of the winter. When brought back into the laboratory, they resumed growth as vigorously as fresh colonies. Desiccation also has no detrimental effect, as shown by the following experiments: In the first one, bark was removed from a canker and stored under perfectly dry conditions in the laboratory. Isolations have been made each month and at present—at the end of ten months—the isolations are just as successful as when the experiment was started. The second was like the first except that diseased wood was stored instead of diseased bark. This has been in progress only six months, but the isolations are still successful. That a pile of bark or chips may be a source of infection for a long time on account of the mycelium is

indicated by the following experiment: One year ago, some diseased logs were peeled and the bark thrown into piles. Isolations have been made from these heaps at the end of every month—being careful to avoid contaminations from spores of the fungus—and up to the present have been entirely successful. The writer has been unable to find any especially resistant cells in the hyphae which tide it over.

The mycelium also invades the sap-wood to a depth of about four or five rings. The hyphae are not different here except that they are smaller than in the bark and do not enter the wood as fans. They grow through and destroy the cells of the medullary rays and wood parenchyma to some extent, and are found in the vessels in abundance, but the walls of the latter are not affected by them.

PYCNIDIA.

The summer spores in all cases are produced in pycnidia. The stages in the development of this organ are most readily observed on artificial media, such as potato agar or chestnut bark agar. The process is the same whether it takes place on agar or under the cork layer of the tree or superficially on the exposed wood. But on agar it is more simple and more easily followed. It will therefore be taken up in detail as it occurs on artifical media, and then more briefly on the bark and on the wood, noting particularly the points in which they differ.

Development on artificial media. The first stages can be watched directly under the microscope in Van Tieghem cells. Cultures of pycnospores are made just as stated previously in describing the methods of artificial germination of these spores. At the end of twenty-four hours they are germinating, and in about four or five days, at summer temperatures, the beginnings of the pycnidia can be seen. They appear first where the weft of mycelium is the thickest but they are more easily followed if one finds them on more isolated branches. At certain points short cells are developed in the hyphae by laying down of new walls, thus dividing the old cells. The cells also increase in diameter and in the amount of cell contents. Each of these short cells now sends out stubby, septate branches, the cells of which in turn send out other branches. Such a stage is shown in figures 1 and 2. By the continued branching—or budding—of these cells, a tuft of hyphae is formed which reminds one of a witches' broom. This tuft seems also to exert an influence on the neighboring hyphae and the more distant branches of the same hypha, because they now grow toward it and mingle with its branches so that in another day or two, the mass of hyphae becomes so dense that a surface view no longer shows what is occurring. The little blocks of agar are then fixed in fixing solution,

sectioned and stained to be studied in cross section. Figure 3 shows a cross section of a pycnidium grown in this way. It is merely a solid ball of hyphae densely intertwined but not grown together in any way by their lateral walls. The hyphae appear to be all alike in every particular, that is, there is no differentiation of wall cells and core cells.

The succeeding stages are best studied by the following method: A single culture is made at the center of an agar plate and permitted to grow until it has almost reached the edge of the plate. Beginning at the center, concentric rings of pycnidia are formed as shown in figure 51. Starting from the outermost, the pycnidia of each ring are one day younger than those of the next succeeding ring. This gives a perfect series of successive stages, from those which are so small that they can barely be seen with the naked eye to fully mature ones pushing out spore horns at the center of the plate. A perfectly flat cross section of one on the outer ring is given in figure 4 and shows that it corresponds to the stage observed in Van Tieghem cells and represented in figure 3. It is merely a solid tangle of undifferentiated hyphae. There is, as yet, no evidence of a cavity at the center. In the next older stage, figure 5, the hyphae begin to pull apart slightly and become loose at the center but are not otherwise differentiated. Those branches which extend into this loose area begin to lay down cross walls at regular intervals and as the cells, thus formed, become mature they are cut off successively from the ends of the hyphae and lie free in the cavity (Figure 57). These short cells are the first pycnospores. As all the branches projecting into the central area are cut up to make spores, the cavity is naturally enlarged. But other branches now push in from the surrounding hyphae and more spores are cut off from their apices until the cavity becomes densely filled with them. The size of the cavity increases then, first, by the constant cutting off of the branches and, second, on account of the increased pressure from within caused by the packing of the spores. Also the crowding for space by the new conidiophores would tend to distend the walls. This pressure from within causes the hyphae which are on the periphery to be crowded together and to form a sort of a wall. This wall layer is not so distinct in the pycnidia on agar because there is nothing on the outside to resist the pressure but in the pycnidia on the bark it is quite distinct. Also, the membranes of the wall cells become somewhat thicker at this time. A section from the wall in this stage, showing the relation of the conidiophores, is shown in figure 6. There is no ostiole whatever at this time but a little later the hyphae become loose at a point on the upper wall of the pycnidium and the spores are forced out through this by the pressure from within. The ostiole is thus formed by the same process as the cavity itself. It is very indefinite

at first but as it becomes older and wider, it becomes surrounded by a more definite wall just like that of the cavity.

When fully mature, the cavity may be as much as a fourth of a millimeter in diameter. It is usually almost circular in cross section, but sometimes shows the convoluted form which will be described later as occurring in mature stromata on the bark. The conidiophores form a dense, brush-like fringe and extend directly out into the cavity from every point of the wall. They are of uneven lengths, the majority being 20-40 microns long and about 1.5 microns in diameter. Four of them are shown highly magnified in figure 7. In an unstained section, the septa of the conidiophores cannot be made out but, when properly stained with iron-alum haematoxylin and erythrosin, the septa show up very plainly as unstained lines across the sporophore. It will be seen that almost the whole length of the conidiophores is divided into regular cells, each of which contains a single nucleus. As the cells become mature, they break off successively as conidia. Just how many break off from a single conidiophores was not determined. The majority of them are simple, but branched conidiophores, as shown in figure 7, are not uncommon. But they are never so frequent or so much branched in this type of pycnidium as in the types to be discussed later. In the older pycnidia they are longer than in the young ones. Among the conidiophores are certain longer branches which project further into the cavity. These are evidently the structures which Pantanelli (89) calls paraphyses. Yet he seems to have some hesitation in designating them by that name, because in a footnote at the bottom of the page he adds; "Non tutte si possono considerare come parafisi o pseudoparafisi, perche talvolta formano conidii alla loro estremita." The writer also found pycnospores on the tips of them and they are also divided into the same regular uninucleate cells as the conidiophores. They branch like the conidiophores and, as for their length, all lengths can be found from 75 microns down to 10 microns. One would be excusable for wondering on what basis they would be distinguished from the conidiophores.

Factors influencing production. As indicated above, the time required for the production of pycnidia on artificial media is very short. When ascospores, naturally ejected from the perithecia, are caught on plates of sterile chestnut bark agar, they germinate in a few hours and at the end of from five to seven days—where they fall thickly on the agar—a pycnidium containing mature spores will be formed at every point where a spore or group of spores fell. These pycnidia differ in no way from those described above. When cultures are made from pycnospores by making streaks on potato agar, pycnidia containing mature spores are usually developed within eight days at ordinary summer temperatures. At lower tempera-

tures, the time required is much longer. As previously mentioned, plates of the fungus exposed to out-of-doors temperatures during the last winter showed considerable growth of the mycelium but in no case were pycnidia produced on these plates. Also on the trees, where the spread of the cankers was measured each month by a painted outline, it was observed that no pycnidia or even "blisters" were developed on the diseased areas that were added during the winter. These experiments indicate that the fungus will grow at a lower temperature than that at which it will produce pycnidia.

Another factor which influences the production of pycnidia is light. When plate cultures are grown in total darkness on chestnut bark agar, no pycnidia are developed, while on plates made at the same time and grown in the light, the usual rings of pycnidia appear (Figure 57). Experiments were also tried in which the plate was left in darkness until about half-covered with mycelium and then brought into the light. Circles of pycnidia were developed, beginning with the ring which marked the outermost limit of the colony when removed from the dark chamber. The concentric rings which always appear on agar cultures are due to the alternation of night and day.

When young trees in the woods are inoculated, the pycnidia do not become evident as soon as on artificial media. But, even here, the spore-horns have been observed in three weeks on inoculations made with pycnospores. "Blisters," indicating the development of the pycnidia under the cork layer, have been observed in eighteen days.

Development of pycnidia on the young canker. The first outward indication of the pycnidia is the appearance of numerous little raised "blisters" just back of the advancing edge of the canker (Figure 45). They are perfectly smooth little mounds and, under the hand lens, appear slick and somewhat translucent. Contrary to published statements of investigators of this disease (e. g. 4: 187), they bear no relation whatever to the lenticels. They seem rather to avoid the lenticels. On account of their smooth, unbroken surface they cannot be confused with the latter at this stage, but at later stages, when they are broken open at the apices, they often give the erroneous appearance of having been formed in the lenticels. They are much more numerous than the lenticels, often being so thick as to be in contact with each other. If the cork layer is carefully removed, the beginning of a single pycnidium will be found under each of these raised places. At this stage they are hyaline, more or less globose or biscuit-shaped cushions with a moist gelatinous appearance, about half imbedded in the disintegrating collenchyma tissue, the other half projecting upward and raising the cork layer to form the pimple. In size, they vary from those on the outermost edge which are almost microscopic to those a millimeter in diameter just

before the breaking of the phelloderm. There is no stroma at this time, but each one is very early surrounded with a fringe of loose mycelium which is the forerunner of the stroma. It is at first white but begins to turn yellow even before the cork layer is broken. When a cross section is made of this moist-looking cushion, it is found to be a closely wound ball of hyphae corresponding to figure 4, as decribed under the development of the pycnidium in culture. There are no pycnospores and as yet no indication of a cavity. From the periphery toward the center of the canker the cushions are successively larger and more of the developing stroma about them until the cushions are entirely covered by the mycelial weft, which is now bright yellow. The cavity, sporophores and pycnospores are developed from this cushion in exactly the same way as described above on agar plates and will not be again described. Where the pycnidia originate very closely together, the stromata often come into contact and coalesce so that we now have a compound stroma—to all appearances, a single stroma containing several pycnidia. This condition has been found by the writer in mature stromata several times but seems to be rather the exception—a single much convoluted or labyrinthiform pycnidium in each stroma being the rule. Apparently, even when by coalescence several pycnidia are thrown into one stroma, the receding walls of the chambers soon come into contact and portions of them are broken down so that there is now one large, irregular cavity. So far as observed, the stroma never precedes the pycnidium. A pycnidium first starts and later the stroma forms about it. There is no rind layer on the stroma previous to the breaking of the cork layer. This latter process is brought about through pressure exerted by the growing pycnidium beneath. By this time the spores have developed and soon push out in curling tendrils through the rent in the cork layer.

Spore Horns. They are light yellow in color at first and have a waxy appearance. As they become older they take on a reddish cast. They vary in size from the diameter of a hair to a half-millimeter and in length from a millimeter to more than 2.5 cm. The writer and J. R. Guyer measured an exceptionally long one that was two and one-half inches in length. On young cankers on smooth-barked trees, they are usually small in diameter, single and twisted into several coils, but on the bark of old trees, where they come from the lines of stromata in the crevices, they are large, stout and irregular and often a whole line of them are united comb-like. Figure 48 shows this condition in which they are coming out from rough, burnt-over bark. In cross section, the horns are usually flat or irregular in shape, and only rarely circular. This accounts largely for the way they curl. The irregular twisting is shown in figure 47. When dry, they are hard and brittle, and it takes some little effort to break

them loose. It is doubtful if a wind is ever strong enough to break them off and carry them away when dry. But when they become wet, they swell and the spores—of which they are entirely composed—separate and wash down the tree, but as soon as the rain is over, new spore-horns appear with surprising rapidity. Just how long a pycnidium will continue to produce spores has not been determined. During the last season, on young cankers produced by inoculation in the spring, the horns were abundant after each rain until the latter part of the summer, when the pushing out of the stromata indicated the beginning of the perithecial stage. After that, very few spore-horns were found on these cankers. Heald and Gardner (93) have shown that the pycnospores are produced in the winter. Except in cases where they were protected and kept dry, so that tendrils produced in the summer were not washed away, the writer has not seen spore-horns in the winter, but this is probably due to the fact that they are produced at such a slow rate that they are washed away before their size makes them noticeable. They first began to appear this season, (1913), about the middle of April.

Pycnidia in the older stromata. About the middle of the summer, on cankers produced by inoculations in the spring, there is an active increase in the amount of stromatic tissue, and the pycnidia in the top of this new stroma are pushed out through the cork layer. Meanwhile they continue to increase in size. During this increase, the cavity does not remain round but becomes intricately labyrinthiform, as shown in figures 11 and 55. This shape is easily explained when one considers the method by which the pycnidium increases in size. As previously indicated, the walls are constantly receding in all directions. The new stromatic tissue is mingled with portions of the disintegrating host tissue, and when the receding wall comes in contact with this tissue, it continues to recede on both sides of it, but the part around the obstruction remains as a process jutting out into the cavity. This is repeated many times until often the entire stroma will be found honeycombed with numerous but communicating irregular chambers. A simple case is shown in figure 55. This explanation accounts for the shape of the pycnidium only in part because this type is sometimes found on agar cultures where there are evidently no such obstructions. When cross sections of the stromata are cut, a single section usually shows a number of cavities which do not appear to be connected, but if the entire stroma is cut into serial sections, it will usually be found to contain but a single many-chambered pycnidium. Occasionally however, the writer has found stromata which contained three or four distinct pycnidia.

The pycnidial form of this fungus has often been referred to the genus *Cytospora,* based on the idea that the stroma typically con-

tains a number of pycnidia. Evidently this is a mistake. If there is need of a distinct generic name for this stage, it should be referred to *Endothiella,* a genus erected by Saccardo, (Ann. Myc. 4:73), based on the imperfect form of *Endothia gyrosa.* Saccardo did not apply this name merely to the superficial type on wood, but under this word he included all forms of the pycnidial stage. The labyrinthiform pycnidium in the mature stroma becomes larger than the forms developed on agar and on wood. Cavities more than a millimeter in diameter have been found by the writer. Besides differing somewhat in shape and size, this type also differs from the type on agar in that the wall layer is more distinct, and the conidiophores are more branched and longer.

Superficial pycnidia. Another form of the pycnidium is found on the cut ends of stumps and logs and both on the wood and the inside of the bark where the latter has broken loose and an air space is left between it and the wood. These are superficial, single pycnidia. A group of them is shown in figure 12. A favorite place for them is on the inside of the bark where it has drawn away from the stump around the top, after the tree is cut. Also after a log or stump on which there was a canker is peeled, the pycnidia will develop on the surface very quickly if it does not dry out too soon. Their production is largely dependent on the water supply. This is illustrated by the fact that in dry weather they will develop on the lower side of a log lying on the ground, but not on the upper side. Their shape also varies with the amount of moisture. In the more moist, shaded situations, they are long pear-shaped or conical, as shown in figure 12, or the base may be flattened out slightly on the substratum. But on tops of stumps—where they occur abundantly on the outermost four or five annual rings, and where the supply of moisture is not constant—they are flattened out on the substratum and do not stand out free as shown in the figure. Also they have more of a tendency to run together here. In color they are deeper red than the stromata, but have light yellow conspicuous ostioles which project upward in a sort of neck or beak. They are surrounded by no stroma whatever, and stand out free so that they can easily be picked off with a dissecting needle. They measure about a quarter of a millimeter in diameter and the same in height. The outer wall is perfectly smooth as seen under the hand lens. Often several of them grow together, but their ostioles remain distinct and we have the appearance of a single pycnidium with several ostioles.

The writer has not seen all the developmental stages of this type, but there is no reason to believe that they differ essentially from those on agar or under the cork layer. A cross-section of one when mature, (fig. 54), shows no differences in the configuration of the cavity, the character of the conidiophores, etc. The walls are thicker and

much more dense, however, and the ostiole is more perfectly formed than in the others previously observed.

Usually, this type of pycnidium is not followed by the perithecia, but in two cases, where they were between the bark and the wood, the writer has found perithecia developing among them.

STROMATA.

The stromata are more often seen and better known than any other stage of this fungus. They are the reddish brown cushions mentioned in the introduction, which are scattered thickly over the canker and make it so conspicuous and easy of diagnosis. A canker thickly beset with them is shown in figure 44. The beginning of the stroma has been mentioned in treating of the pycnidium. As stated there, it always starts as a loose growth of hyphae around the pycnidium. It does not precede, but follows the first stages in the development of that organ. This stage of the stroma may often be observed on agar cultures where the pycnidia are rather far apart. A fluffy growth of light yellow mycelium surrounds the pycnidium, and covers it over until often nothing can be seen but a mass of spores oozing from the top of a loose ball of hyphae. If these are imbedded and sectioned, they will be found to contain a loose tangle of undifferentiated hyphae surrounding a central pycnidium. No rind layer is produced under these conditions. This corresponds to the stage on the bark which precedes the rupturing of the cork layer. But as soon as the cork layer is broken, the stroma undergoes a change. There is a rapid increase in size, and at the same time, a differentiation of the cells at the tips of those branches which reach the exposed surface. These cells now become shorter and thicker, acquire heavier walls, and are densely crowded together, so that in cross section they appear as a pseudoparenchymatous tissue (Fig. 10). The rind thus formed covers all of the exposed surface of the stroma, and also grows up around the necks of the perithecia (Fig. 11). The cells are pretty well filled with protoplasm and stain deeply. They also contain more pigment than the other cells. The interior or medulla of the stroma remains the same. As shown in the base of figure 10, it is merely a loose tangle of hyphae which are much branched and more often septate, but in all other respects, like the usual vegetative hyphae. The cell contents, nuclei, vacuoles, walls, etc., are just the same. They also contain a large amount of pigment. Stone cells, bast fibres and remnants of the walls of the collenchyma cells are scattered through the basal parts. A diagrammatic drawing of a stroma showing the location of the pycnidium, perithecia and rind layer is given in figure 11. When they first come through the cork layer, they are lemon yellow in color but with age the color deepens to orange, reddish brown and finally

cinnamon brown, But when cut into, they are found to be lighter colored on the inside than on the surface. Fully mature, they average about 2.4 x 1.2 millimeters in size, being usually elongated horizontally as shown in figure 44. They average about 1.3 millimeters in depth. The size however, depends largely on the location and the season. If they grow in a moist situation they are much larger than where they are exposed to desiccation. On old rough bark, they do not occur as shown in figure 44, but come out only in the crevices of the bark, often united in a solid line for several inches so that they apparently form one long stroma. Otherwise they do not differ from those described above.

PERITHECIA.

Previous to the beginning of the perithecial stage, the cork layer has been broken only by the emerging spore-horns. The small amount of stroma that is developed lies entirely beneath this cork layer, that is, none of it is erumpent as yet. The change to the perithecial stroma has been observed within eight weeks after inoculation. On trees inoculated in June the stromata have been observed in August. The stroma increases very rapidly in size and pushes off more of the cork layer. Not only does it fill up the enlarged rent in the phelloderm, but it also grows out over the torn edges to some extent so that they are included in the stroma as shown in figure 11. If one peels off the cork layer now, either the entire stroma, or at least the top comes off with it. The stroma now has an erumpent superficial appearance as shown in figures 43 and 44.

Primordia. When we speak of the perithecial stroma, however, we do not mean that it contains perithecia as yet. Spot infections have been under observation where the perithecial stromata were in abundance on all the cankers in the early spring, but there was no outward appearance of perithecia during the entire summer. On the other hand pycnospores may be pushed out from these stromata in numerous spore-horns during the entire season. Cross sections of these stromata show that the pycnidia are now located in the periphery, the mass of stroma having been formed beneath them and pushing them out through the cork layer. Their location is shown in figure 11.

The most noticeable feature in a cross section at this stage is the numerous primordia—the earliest stages in the development of the perithecia. These arise usually in the tissues of the bark below the base of the original pycnidium and by their growth and the growth of the new stromatic tissue about them, they push these disorganized elements upward and apart so that scattered fragments of them are found included throughout the base of the stroma. The primordia do not always originate however in the lower layers. At times they

may be found well up in the stroma without a trace of the disorganized bark about them. A stained cross section shows one or two very prominent large, deeply stained cells at the center of each primordium, and running around these in close concentric circles are enlarged strands of mycelium. These latter also stain quite heavily so that the stain may be taken out of all the rest of the stroma and still leave the primordia quite prominent.

The number of primordia in a single stroma may be very large—over one hundred having been counted in one. They fill up most of the available space in the base of the stroma and are often so close that they give the appearance of double or triple primordia. All of them however, do not develop into mature perithecia on account of the lack of space and possibly of food supply. When the perithecia are mature there are usually fifteen to thirty in a stroma. This means that one out of every four or five primordia reaches maturity. Their degeneration takes place at all stages almost up to the mature perithecium, but by far the greater number never get past the ascogonial stage. Sections of the stroma at any subsequent stage will show these starved primordia in the base. Both the ascogonial cells and the enveloping hyphae lose their contents almost entirely, and appear as empty cells which no longer take the stain like those of the healthy primordia and are usually pressed out of shape by the growth of the latter.

The large central cells are part of the organ which was first known as the Woronin Hypha but now more commonly called the carpogonium. The cells of the carpogonium lying within the enveloping hyphae as described above are the ascogonial cells, or simply the ascogonium. In a thin section usually only one or two of them is seen, (Figs. 19 and 20), but if serial sections are examined, it will be found that they number from two to five in each primordium and are wound into a circle or, more often, a spiral of one or two coils. Occasionally, the entire structure may be seen in one section as shown in figure 21. The cells are elongate, oval and slightly curved to fit into the segment of the spiral of which they are a part. Fully mature, each measures about 10 x 25 microns. They are deeply constricted at the septa and apparently are only loosely connected; in fact in prepared sections they are very frequently not in contact at all—especially the older ones.

They are very densely filled with protoplasm, and for this reason, easily brought out by differential staining, retaining the protoplasmic stains with great tenacity. They are best stained with Heidenhain's iron-alum haematoxylin and erythrosin. The nucleoli are especially tenacious of the haematoxylin, and in a properly differentiated cell, the writer has counted as high as eighteen nuclei. They may be quite readily brought out by Flemming's triple stain. These two

stains have been used interchangeably, their relative efficiency depending on the points to be brought out and the stage under consideration. Outside the nucleolus, however, the resting nucleus does not retain the stain when treated with the haematoxylin and a definite nuclear membrane is made out only in the more favorable cases. The usual appearance of the nucleus is shown in figure 20, merely an intensely stained nucleolus surrounded by a circular clear area. The nuclei are much more numerous in the ascogonial cells than in the cells of the enveloping hyphae, usually only about two to five appearing in each of the latter. They are also larger and more prominent.

The ascogonial spiral does not terminate inside the primordium but is continued up through the stroma as a large-celled, prominent, deeply staining thread. The thread can be traced entirely to the surface of the stroma. The cells are of a less diameter than in the cells of the ascogonium and not curved and do not show such deep constrictions at the septa. The cell contents, including the prominent nuclei, are the same as in the ascogonium. Fourteen nuclei have been counted in a single cell. This thread has been called the trichogyne and the writer will continue to use that term, not implying by so doing that it has the functions of a true trichogyne. They are often found branching, and in the upper part of the stroma they may be distinguished in great numbers on account of their avidity for stains. It is not so easy to trace them through the pseudoparenchymatous rind because the cells of the latter are quite compact and stain deeply. The apical cells usually project slightly beyond the surface.

So far as could be determined, the trichogyne is a useless organ in the development of the perithecium. It is probably a remnant of an ancestry in which a copulation with a free spermatium was essential to the further development of the carpogonium. Lindau* has suggested as the function of a similar organ in the lichens the breaking of a way through the thallus for the emerging apothecium. A similar function here, that is, making a path for the advancing neck of the perithecium, is very doubtful. The trichogyne threads become less distinct as they become older and finally cannot be seen any more.

The stage containing the mature ascogonia is evidently a resting stage for it has been found more numerously than any of the other developmental stages of the perithecium. As a rule, the primordia of one stroma are all in the same stage. The writer hoped to find stromata in which the primordia were all in a younger stage, in which he could determine the exact origin of the ascogonium. Up to the present however, he has not secured such a stroma, and has

*Lindau, G. "Uber Anlage und Entwicklung einiger Flechten Apothecien." Flora, 1888.

had to depend on a relatively small number of apparently incipient primordia which were found in older stromata. The earliest stages found are represented in figures 15, 16 and 17. They show merely a coiled hyphal branch, somewhat larger than the stromatal hyphae which surround it and taking the stain very deeply. In figure 15 there is no indication of a differentiation of the surrounding hyphae to form the envelope. Figures 16 and 17 show the beginning of such a differentiation. Whether this young ascogonial branch is a new formation, or whether it is merely a transformed pre-existing branch of the mycelium, could not be determined with certainty, but the writer is inclined to the latter view by what evidence he has seen. The envelope is differentiated from the surrounding hyphae, and is in no direct connection with the ascogonial branch. As the ascogonial cells increase in size, the number and size of the enveloping cells also increases as indicated by the succession shown by figures 16, 17, 18, etc.

Degeneration of the ascogonium and growth of the enveloping hyphae. Figure 21 shows the highest point of development in what we have called the ascogonial stage. The entire primordium is now about 50-75 microns in diameter. The material from which this figure was drawn was taken in the late fall. In the first week of the following March, material was collected from the same tree, and all the primordia now appeared in cross-section like figure 22. This is the beginning of a new stage of development. The seat of activity seems to have been removed from the ascogonium to the enveloping hyphae. From this time on, the ascogonium degenerates. The dense protoplasmic content gradually disappears, and now the contents are represented either by ragged bridles across the lumen and irregular masses around the walls, as shown in figure 22, or else the entire contents draws up into a misshapen mass which stains very deeply with safranin.

The behavior of the enveloping cells is quite the contrary. Their contents now becomes more dense and retains the protoplasmic stains more deeply than the ascogonial cells. Their nuclei also become more prominent and apparently more numerous. Up to this time the individual hyphae can be traced, and there are open spaces between them; but now they have increased both in size and in number, and filled up the intervening spaces. They appear as a pseudoparenchymatous tissue instead of a coil of hyphae. The increased growth presses in the sides of the ascogonial cells which now have nothing within to keep up their turgor.

The most important question at this time is in regard to the branching of the ascogonium. Reasoning from analogy with many other Ascomycetes, we would expect the ascognia to give rise to ascogenous hyphae before their degeneration. Many hours were

spent searching for these hyphae. Only in a few cases was a condition found which would lead one to believe that there were such branches. Three of these cases are shown in figures 24, 25 and 27. All of these, however, occurred when the ascogonium was about ready to break down. A distinct opening between the ascogonia and these cells could be made out. The cells of these "apparent branches" differ little from the surrounding cells except that the first cell is usually almost devoid of contents, like the ascogonium. Since there is no way of distinguishing them from the surrounding cells, their identity cannot be determined in subsequent stages. In the vast majority of cases, no such branches were found, but this may have been due to a lack of sufficient material in the right stage for observation of this point.

Beginning of the differentiation. The primordium now increases very rapidly in size. The cells at the center grow more rapidly than those at the periphery and at the same time the contents become more vacuolar. The reciprocal pressure gives them more and more the appearance of a pseudoparenchymatous tissue. The peripheral cells on the other hand become elongated and flattened by the pressure from the center, and at the same time are less vacuolar than the central cells. This stage is shown in figure 23. As yet there is no sharp differentiation of the wall cells. The crushed remains of the ascogonium are occasionally seen at this stage but have not been found later.

This period also marks the beginning of the neck, which is initiated by a vigorous outgrowth of small cells at a point of the periphery toward the exposed surface of the stroma, forming a blunt cone (Fig. 23). The cells are very compact and have a dense protoplasmic content with several small nuclei in each cell. It is not possible at this time to trace individual hyphae in the young neck. No canal is evident.

The next step marks a complete differentiation of the core cells and the cells which are to form the wall of the perithecium. The cells at the center become larger and still more vacuolated. The membranes remain very thin. They form a perfectly spherical core and are set off by an even line from the wall cells which have now become more distinctly elongated and flattened. The membranes of the latter cells become thicker and the contents still remain dense so that it is now easy in stained sections to tell the exact dividing line between wall and core. The distinctness of this line gives the impression of two different tissues. A camera lucida drawing of a few cells on either side of this line is given in figure 28. It will be noticed here that one of the cells seems to be differentiating into a core cell at one end and a wall cell at the other. Such a condition indicates that these two tissues are not of different origin. The

core now measures about 135 microns in diameter and the wall is composed of eight to twelve layers of cells and is about 35 microns in thickness.

Pathological conditions. Peculiar pathological conditions of the young perithecium are numerous at this as well as previous stages. The delicate-walled core cells break down very easily and primordia containing a central cavity, even before the beginning of the neck, are common and misleading to any one searching for the normal beginning of the cavity. Frequently very fine hyphae are found entering between the corecells and apparently living parasitically upon them, causing them to break down and thus furnish a rich pabulum for the invading hyphae. Soon a dense, deeply stained tangle of these hyphae fills the lower part of the cavity. These are not the ascogenous hyphae, as the writer suspected when he first saw them, and such perithecia develop no further but may often be found crushed out of shape between the naturally maturing perithecia.

The cavity and paraphyses. The normal formation of the cavity appears about the time the length of the neck equals the diameter of the perithecium. A portion of the cells in the lower part of the core—not on the periphery of the core but inward by about two to four layers of cells—begins to break down, and in this cavity are now found only scattered, irregular masses of protoplasm, degenerated nuclei and occasionally a part of a wall. Sometimes an entire cell may remain intact even after all the cells about it have broken down. But there is never a large cavity at any one time. As soon as a few cells are broken down, the cells which border on the cavity below begin a new period of activity. Even at this time they can be distinguished by more prominent and numerous nuclei; the walls are more distinct and the contents increases slightly in density. These are the initial cells of the paraphyses which are now pushed out into the cavity and follow its receding upper limit. Their origin is shown in figure 29. They very soon become septate and at subsequent stages their origin would be hard to determine. They are composed of short, plump cells, very rich in protoplasm, staining very deeply, and containing several nuclei. The paraphyses branch frequently and are very crooked, and, hence difficult to trace individually in thin sections. Not only do they extend upward into the cavity, but some of them run around the periphery and send out frequent vertical branches into the cavity. They line only the bottom and never come from the roof, at which place the core-cells remain intact for a long time. A perithecium in a rather young paraphyses stage is shown in figure 30. It is now about 200 microns in diameter. There are no ascogenous hyphae or young asci at this time. The outer wall has become more pronounced and is distinctly divided from the bases of the paraphyses by several layers of large, clear core cells.

As the paraphyses become older, their component cells become more elongated and slender. When the young asci appear they begin to lose their dense contents and are soon not easy to distinguish. But even after the first asci are mature, they may be seen as slender filaments devoid of contents except for the nuclei, which persist for a long time. Their function is probably to nourish the growing asci.

The asci. The writer was unable to determine the origin of the ascogenous hyphae. The young asci arise as branches of a system of hyphae which appear among the bases of the paraphyses, but which cannot be distinguished from the paraphysogenous hyphae by staining reactions or otherwise. They are undoubtedly a different system and in no case has an ascus and a paraphysis been seen coming from the same hypha. At the time the asci first appear the perithecium is about 250 microns in diameter, and the neck is nearing the surface of the stroma but has not yet begun to turn black. So far as could be determined from the material examined, the asci arise as ordinary lateral or terminal branches. The young ascus is broadly clavate. In the uninucleate stage, the protoplasm is gathered about the large nucleus, which is usually at the center, the ends being less dense and therefore taking less stain. By three successive divisions, eight nuclei are produced and the protoplasm about them becomes clear and is soon closed off from the epiplasm by a membrane. But, at the same time, the nucleus is dividing again and by the time the wall can be distinguished, there is also a distinct septum in the spore. This condition, in which there is a single nucleus in each end of the spores, does not persist very long but soon there is another division, making two nuclei in each end and frequently, by successive divisions, the mature spore has three or four nuclei in each end, as previously stated. The details of the nuclear divisions and the cutting out of the spores in the ascus, being purely cytological and outside the scope of this work, were not followed more closely.

Mature asci with the spores in place are shown in figures 34, 35 and 36. The arrangement of the spores in the ascus is irregularly uniseriate or subbiseriate. There is, however, no uniformity in their arrangement and two asci can hardly be found in which the spores are placed alike. The epiplasm is still very distinct, especially where it tapers to a point at the top of the ascus. There is a thickened ring—reminding one of a doughnut—about the upper extremity of the lumen of the ascus which is very prominent and shows peculiar staining reactions. It has been suggested that it is at this point that the top of the ascus breaks off to free the spores. This explanation is at least, plausible, but the writer has never been able to find the asci in the process of liberating the spores, and is therefore, unable to confirm the theory. When the ascus is lying flat on the side—as is practically always the case in water mounts, the ring appears in

cross section as two highly refractive disks such as is shown in figures 35 and 36. As figure 34 shows, the spore-bearing part of the ascus is only about three-fourths of its total length. But in dried specimens the point draws down until the ring is very close to the spores as shown in figure 36. The natural shape is not recovered at once on placing the ascus in water. This fact should be taken into account in making measurements. It is best to use only fresh specimens. Murrill (4), gives the dimensions of the ascus as 45—50x9 microns. The average of one hundred and fifty measurements made by the writer was 51.2 x 8.9 microns.

Development of the neck. Even before the complete differentiation of the core- and wall-cells, it is noticeable that the cells on the upper side are pushing outward in a sort of a knob, and by the time the core has become distinct, this structure has become a definite cone as represented in figure 23. At this time the cells are small and very compact, and distinct hyphae cannot be made out. The cone is a perfectly solid mass, that is, there is no indication of a canal in the center. But as the hyphae elongate toward the surface of the stroma, they become less entangled, running almost parallel, converging toward the apex of the advancing cone and leaving an open canal through the center. This advancing apex is shown in figure 31. The hyphae, are slender, very densely filled with protoplasm and, therefore, stain quite deeply. The arrangement is loose and individual hyphae can be traced for long distances. The septa are far apart. The converging apices are usually somewhat swollen. As the apex pushes toward the surface, the stromatic hyphae are not destroyed but are merely wedged apart to make room for the neck. At a distance of about 50-75 microns from the apex, it will be noticed that the hyphae are increasing in diameter and new branches are being inserted. This process continues until the wall of the neck is composed of densely packed hyphae and is quite firm. The walls of these cells also become thick, and about the time the apex has reached the surface, they become black. The apices of the branches which extend into the central canal, however, do not take on these latter characters but remain thin-walled and loose. These are the periphyses. They extend outward and upward and their apices almost come into contact. They are shown in figure 32. They are confined to the neck and never occur within the perithecium proper. But as yet the canal in the upper part of the neck is separated from the cavity of the perithecium by the upper wall of the latter and the cells of the solid cone which formed the beginning of the neck. About the time that the paraphyses are maturing in the cavity, the cells in a direct line from the cavity to the upper canal begin to draw apart and to react differently to stains. These cells have not become thick-walled like the other cells of the perithecial wall. There is prob-

ably also a disintegration of some of the cells which formed the perithecial wall, but not of the cells of the original cone. These latter merely draw apart, and the cells left projecting into the canal thus formed take on the character of periphyses. Also where the canal breaks through the wall, some of the cells are left projecting like periphyses. These periphyses in the lower part of the canal differ from those in the upper part in their irregularity, and in not projecting upward at an acute angle. An early stage in the formation of the lower canal is shown in figure 32.

It is impossible to tell whether the neck follows the course taken by the trichogyne up through the stroma since the trichogyne has entirely disappeared by this time. The stroma is usually much broader at the bottom than at the place where it breaks through the cork layer. For this reason the necks seem to converge at the top. The way in which the necks bend to get through the cork layer is shown in figure 53. Where a broad stroma has formed, however, and a large area of the cork has broken away, the necks extend almost straight upward. There is not naturally a distinct valsoid disk in which all the necks converge. The arrangement is diatrypoid rather than valsoid. This fact is of importance in placing the species in its proper genus. The neck does not usually end flush with the stromatic surface, but extends beyond as a little papilla (Fig. 11). The distance to which the papilla extends depends largely on the location of the stroma and the conditons under which it grows. In a dry situation with plenty of sunlight, it may hardly project at all, while in shaded places and especially where it is moist, it may project more than a millimeter. Much longer ones may be produced by developing them in moist chambers. These papillae are not composed entirely of the hyphae which grow out from the wall of the perithecium but as they push out beyond the surface, the rind tissue grows up about them. A cross section of a papilla is shown in figure 33. If the advancing apex of the neck encounters a pycnidium in the stroma, it grows directly through it or occasionally may curve slightly around it

The mature perithecium. When mature, the perithecium measures about 350-400 microns in diameter and is mostly spherical in shape but the shape is often modified by pressure of other perithecia. As seen under the hand lens, the wall is gray or lead colored but not jet black and shining like the wall of the neck. In cross section, the wall now appears thinner than when the perithecium was young, and the cells are more flattened. The cell-walls are heavy. The structure of the perithecial wall is shown in figure 30. The layers of large core cells which previously divided the contents of the cavity from the wall, have now entirely collapsed and, as a result, the ascus mass is only loosely attached to the wall, and usually pulls away in

sectioning. The entire cavity is now tightly packed with asci. The older ones, having been pushed up are at the center and in the upper part, and the younger ones lining the walls. The writer has calculated the number of asci in a full perithecium at 3600, or 28,800 spores.

Ejection of the spores. Rankin (59) has discovered that the ascospores are forcibly ejected from the necks of the perithecia into the air, and showed that this occurs only during periods of rain. Heald and Gardner (76, 93) demonstrated the effect of temperature, showing that expulsion does not take place below 52° F., and that after being subjected to lower temperature, it requires three or four days of favorable weather to cause further ejection. The writer and Babcock (95) studied the phenomena of ejection with especial reference to its bearing on dissemination. The most essential factor in producing ejection was found to be an abundance of moisture. Under the hand lens it will be noted that there is a film of water over the tip of each active ostiole, and that at each discharge this film is broken and usually eight spores are shot outward, that is, the contents of one ascus. What causes these asci to leave the body of the perithecium and come up to the mouth of the neck was not determined at that time.

If a fresh stroma containing mature perithecia is cut across with a razor, the cut surface will remain level except where the perithecia were cut through. Here the viscous contents will bulge out in a prominent bead, showing that there is a tension inside the perithecium. This is the force which drives the asci up through the canal. There are at least three factors which aid in producing this pressure: (1) The asci do not all mature simultaneously. Young ones are continually pushed up between the bases of the older ones. As they become mature they are pushed up into the center and upper part of the cavity which is soon densely packed, and new ones are still pushing for space. The remaining layers of core cells are first pressed out flat against the walls. (2) But when they would tend to pass out the canal of the neck, the periphyses act as so many little springs and press them back. (3) The most immediate cause of the outward pressure, however, is the swelling of the asci themselves when they become moist. Figure 34 represents an ascus which has been kept in water for several hours. When it is dry, the ascus wall is drawn so tightly up around the spores that it can hardly be distinguished at all except at the top. Figures 35 and 36 show stages of this process. The entire structure occupies less than half the space occupied by the distended ascus. Thus the sudden addition of water, tending to double the volume of the perithecial contents, would easily drive the asci up the neck to the surface. Prepared sections of perithecia which were fixed during the process of ejection, showed that up to the tip of the neck the spores are still in the

ascus. Since the asci are never ejected into the air, it follows that they must burst and liberate the spores when they arrive at the surface film at the tip of the neck.

SUMMARY OF RESULTS.

1. Each pycnospore contains a single nucleus which divides several times before germination, and a polar body at each end. The ascospore contains from one to four nuclei in each cell.

2. Ascospores germinate readily in water; pycnospores require a nutrient medium. Pycnospores germinate on twigs of a large number of common forest trees. They also germinate in humus about the base of the tree.

3. At summer temperatures, pycnospores germinate in 12-36 hours; ascospores in 2-12 hours. Lower temperatures retard germination.

4. Both kinds of spores swell greatly before germination.

5. Pycnospores usually germinate by two tubes and ascospores by four.

6. Ascospores in the perithecia and pycnospores in the "horns" retain their power to germinate at least a year. The longevity is diminished when the spores are separated from each other and when exposed to the air.

7. Winter weather conditions do not affect the vitality of either kind of spores.

8. The cells of the mycelium are multinucleate under all conditions. They are densely filled with protoplasm when young but become vacuolated as they become older.

9. The mycelium and pycnospores are colored by a yellow pigment belonging to the aurine group of compounds.

10. The mycelium does not invade the living tissue as individual hyphae, but in flat fan-shaped mats.

11. The mycelium continues to grow in the bark even during the winter months but much more rapidly in the summer. Its vitality is not affected by winter temperatures.

12. The fungus may be carried over in the bark for a year or more by the mycelium even when the bark is kept dry.

13. The pycnidium is produced symphiogenetically. In the simplest type it is merely a loose tangle of hyphae, the central branches of which become the sporophores. It has a indefinite ostiole.

14. The sporophores are branched and the pycnospores are produced successively from their tips.

15. Pycnidia are not produced in the absence of light.

16. The pycnidium is started before the stroma is formed. It occurs directly under the cork layer and bears no relation to the lenticels. The stroma is formed about the pycnidium and typically there is but a single pycnidium in each stroma.

17. Stone cells, bast fibers and walls of the collenchyma cells are contained in the basal parts of the stroma.

18. The perithecia are produced at the base of the stromata in which the pycnidia are contained.

19. The beginning of the perithecium consists of a coil of large cells—the ascogonium—surrounded by "enveloping hyphae." The ascogonium is continued up to the surface of the stroma in a prominent trichogyne.

20. The trichogyne is not functional as such.

21. The perithecium is differentiated from the "enveloping hyphae."

22. The cavity is formed by the breaking down of the core cells.

23. Paraphyses grow out from the wall into the cavity and almost fill it. They have almost disappeared when the asci are mature.

24. The asci arise as branches of hyphae among the bases of the paraphyses.

25. The neck of the perithecium is produced by an outgrowth of the hyphae on the periphery of the forming perithecium.

26. The spores, still in the asci, are forced out of the body of the perithecium and up to the tip of the canal by (a) the continued growth of young asci from the walls, (b) the swelling of the asci when they become moist.

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106. '13 Fairchild, D. The discovery of the chestnut bark disease in China. Science 38:974-295.

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NOTE.—Many titles were added to original proof sheets.

EXPLANATION OF PLATES*

PLATE I.

Figs. 1, 2. Initial stages in the development of the pycnidium, x 230.

Fig. 3. Cross section of a pycnidium on agar before the beginning of the cavity. x 400.

Fig. 4. Same as figure 3 but a little older. x 430.

Fig. 5. Beginning of the cavity in the pycnidium. x 430.

Fig. 6. Section of pycnidial wall showing conidiophores. x 430.

Fig. 7. Conidiophores. x 800.

Fig. 8. Mycelium from agar. x 800.

Fig. 9. Section of a ray from the fans in the bark. x 430.

Fig. 10. Section of a stroma showing the rind layer. x 600.

Fig. 11. Diagrammatic drawing of a stroma showing the relation to the cork layer and of the organs to each other. x 25.

Fig. 12. Superficial pycnidia. x 14.

Fig. 13. Section of a germinating pycnospore. x 700.

Fig. 14. The resting pycnospore. x 3500.

PLATE II.

Fig. 15, 16, 17, Initial stages of the carpogonium. x 650.

Fig. 18. to 21, Later stages of the ascogonium. x 650.

Fig. 22. Degeneration of the ascogonium and growth of the enveloping hyphae. x 650.

Fig. 23. The young perithecium and the beginning of the stage of differentiation. x 650.

Fig. 24, 25, 27, Apparent branching of the ascogonium. x 650.

Fig. 26. Degeneration of the tricgogyne cells. x 650.

PLATE III.

Fig. 28. Wall and core cells. x 650.

Fig. 29. Beginning of the paraphyses. x 650.

Fig. 30. Perithecium in the young paraphyses stage. x 230.

Fig. 31. Advancing tip of the neck. x 500.

Fig. 32. Lower part of the canal in the neck. x 460

Fig. 33. Cross section of papilla showing periphyses in the neck. x 260.

Fig. 34, 35, 36, Asci showing stages of drying up. x 650.

*All drawings made with the aid of camera lucida except 11 and 12.

Fig. 37. Mature ascospores. x 900.

PLATE IV.

Fig. 38. Outline drawings of germinating pycnospores.

PLATES V AND VI.

Fig. 39. Germination of pycnospores.
Fig. 40. Germination of pycnospores.

PLATES VII AND VIII.

Fig. 41. Germination of ascospores.
Fig. 42. Germination of ascospores.

PLATE IX.

Fig. 43. Canker showing atrophy.
Fig. 44. Canker showing stromata.

PLATE X.

Fig. 45. The blister stage.
Fig. 46. Stromata showing papillae, indicating the perithecial stage.

PLATE XI.

Fig. 47. Spore horns on smooth bark.
Fig. 48. Spore horns in crevices of rough bark.

PLATE XII.

Fig. 49. Canker outlined with paint to indicate monthly growth.

PLATE XIII.

Fig. 50. Mycelial fans under the bark.

PLATE XIV.

Fig. 51. Rings of pycnidia on chestnut agar cultures.

PLATE XV.

Fig. 52. Photomicrograph of pycnospores.
Fig. 53. Vertical section of a perithecium.

PLATE XVI.

Fig. 54. Photomicrograph of pycnidium on wood.
Fig. 55. Stroma containing labyrinthiform pycnidium.

PLATE XVII.

Fig. 56. Vertical section of stroma showing empty perithecia and the black necks.
Fig. 57. Vertical section of young pycnidium on agar showing early stage in the formation of the cavity.

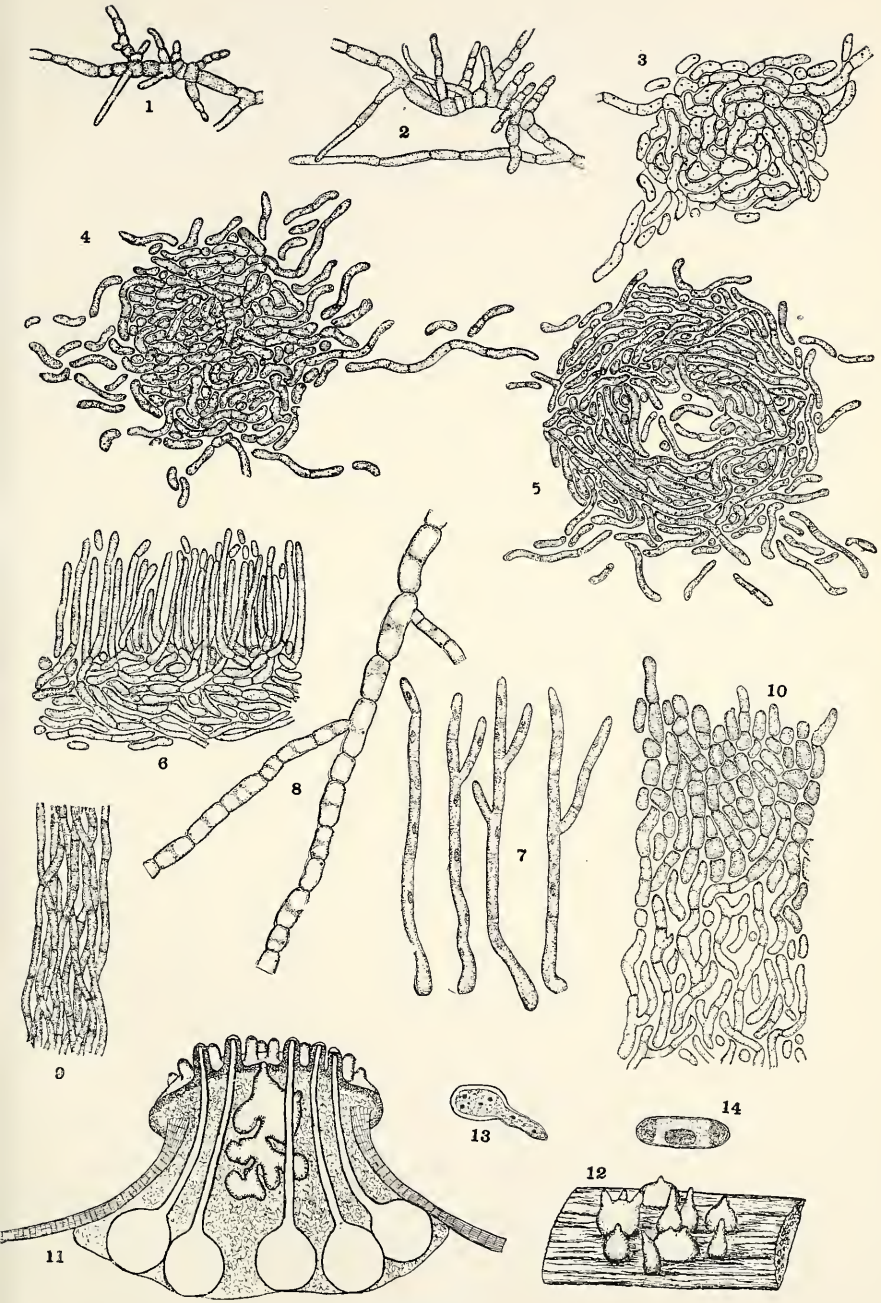

PLATE I.

Development of Pycnidium.

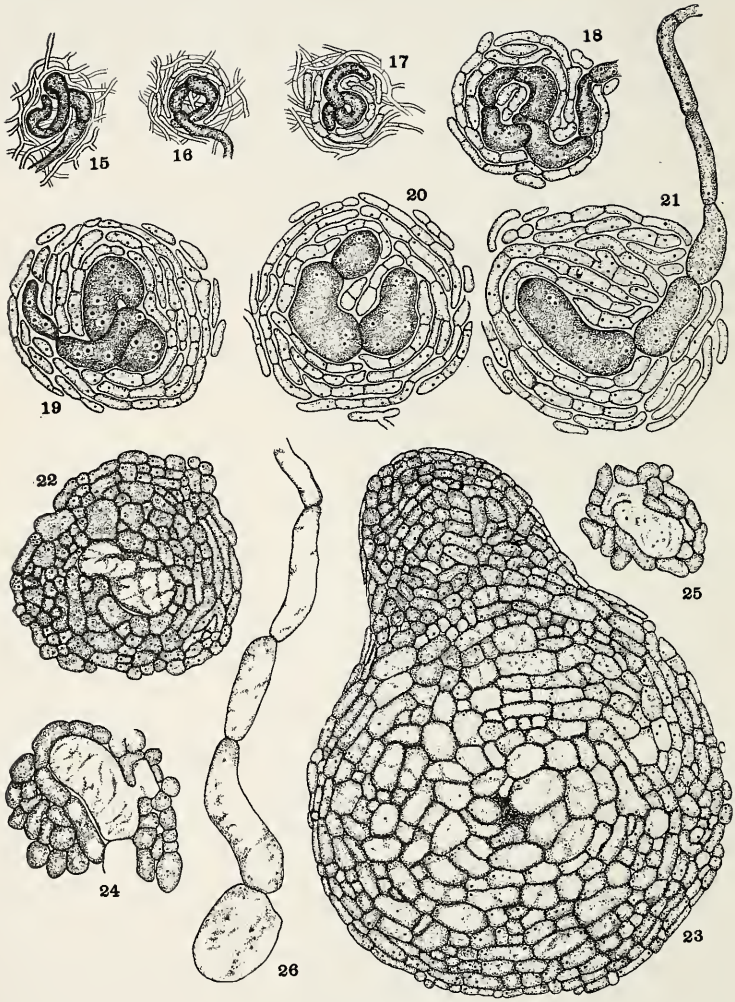

PLATE II.

Development of Perithecium.

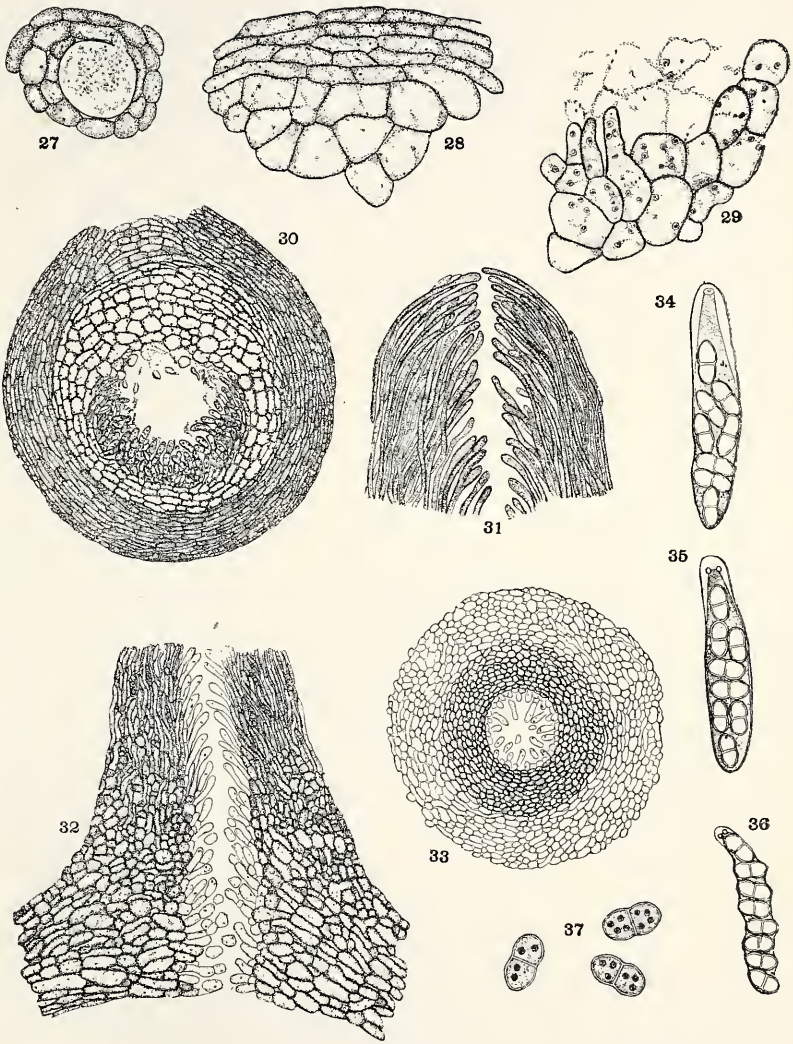

PLATE III.

Development of Perithecium.

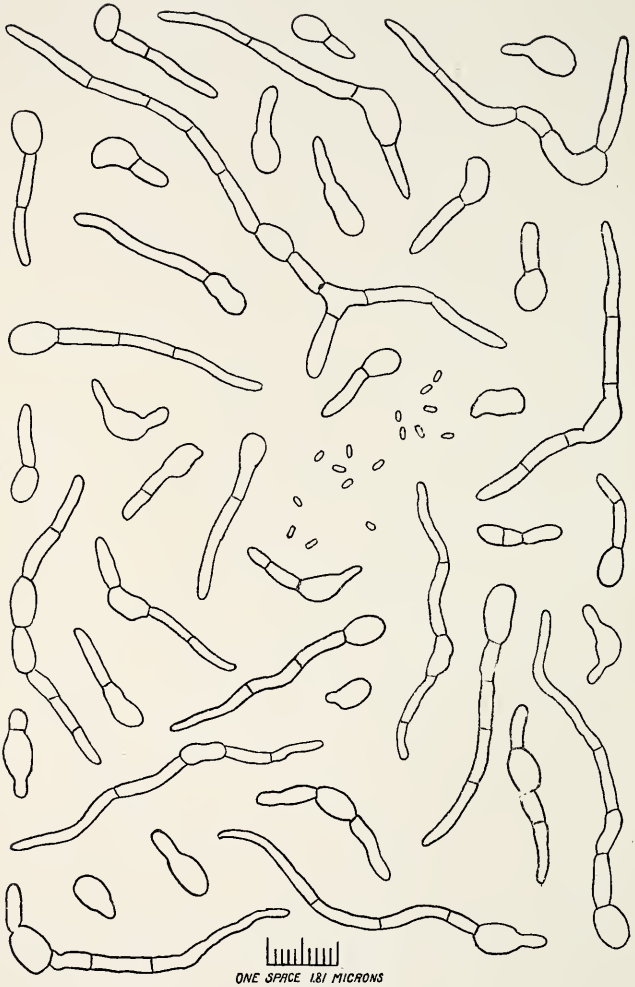

PLATE IV.

Germinating pycnospores

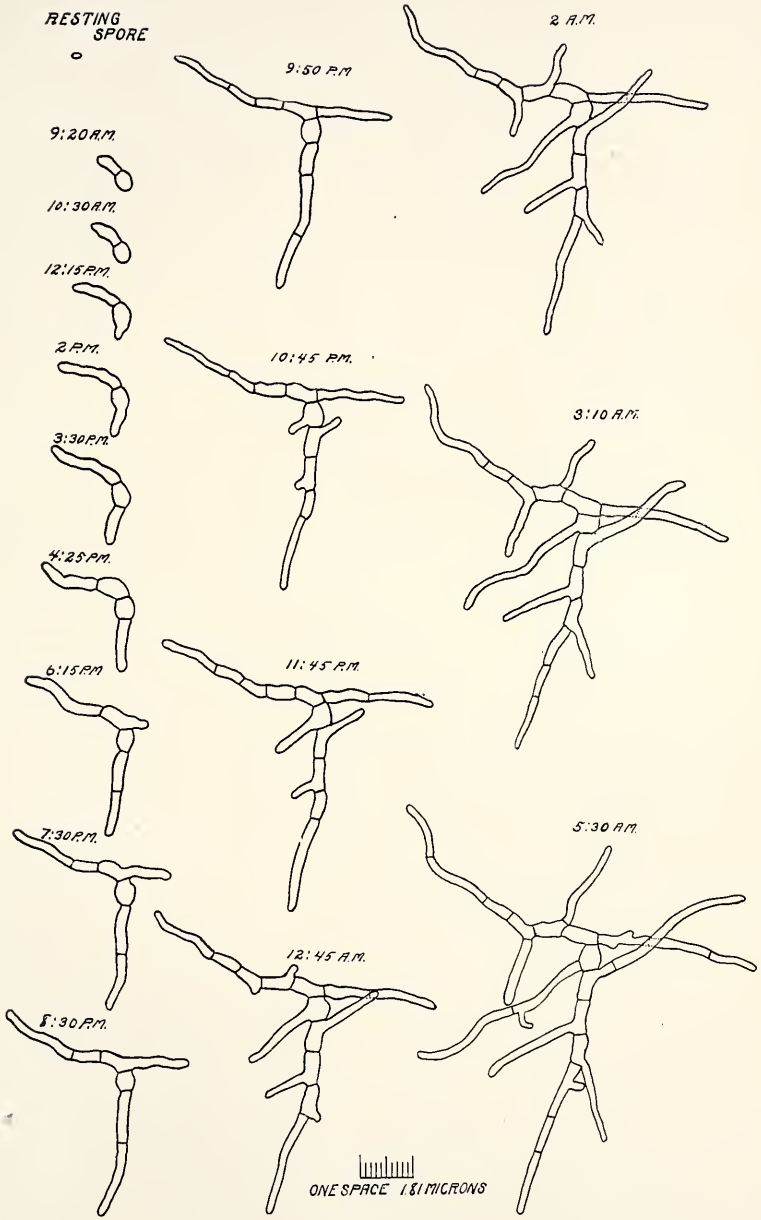

PLATE V.

Germination of pycnospores.

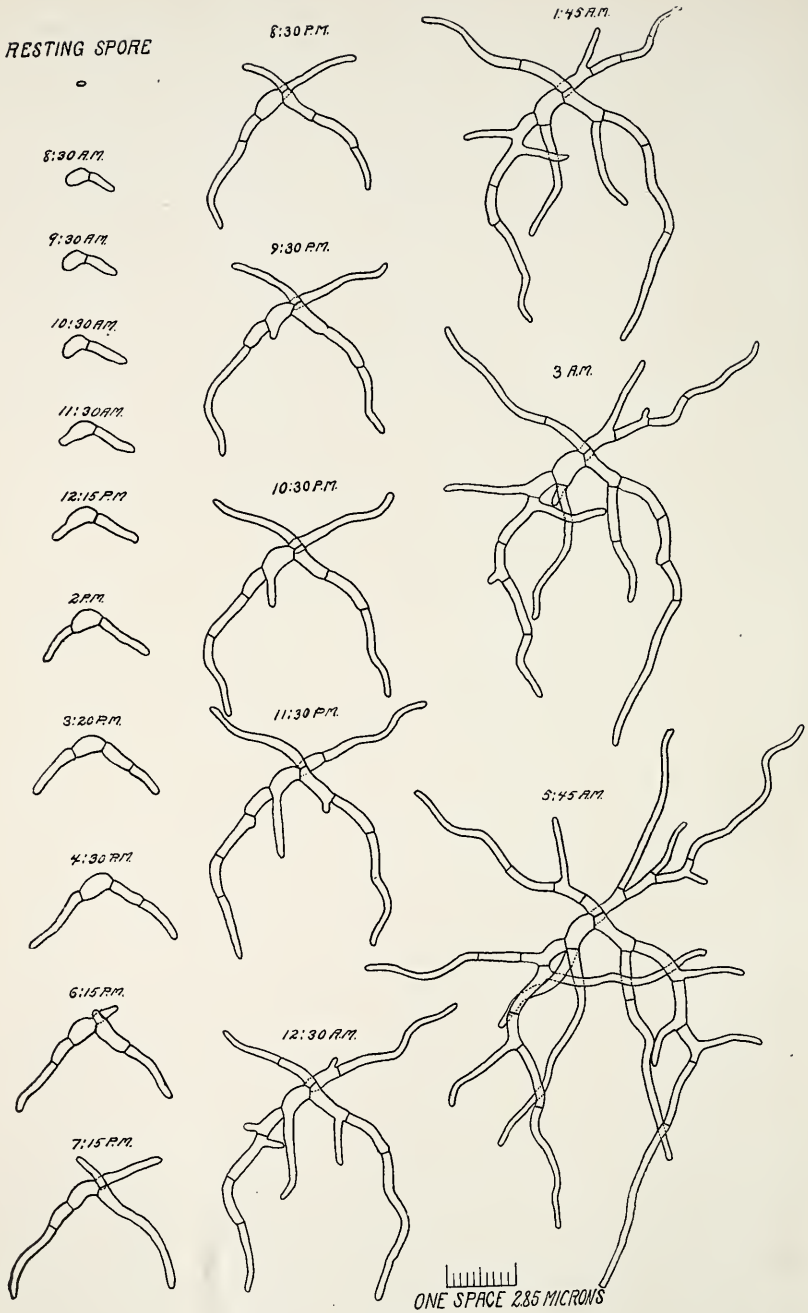

PLATE VI.

Germination of pycnospores.

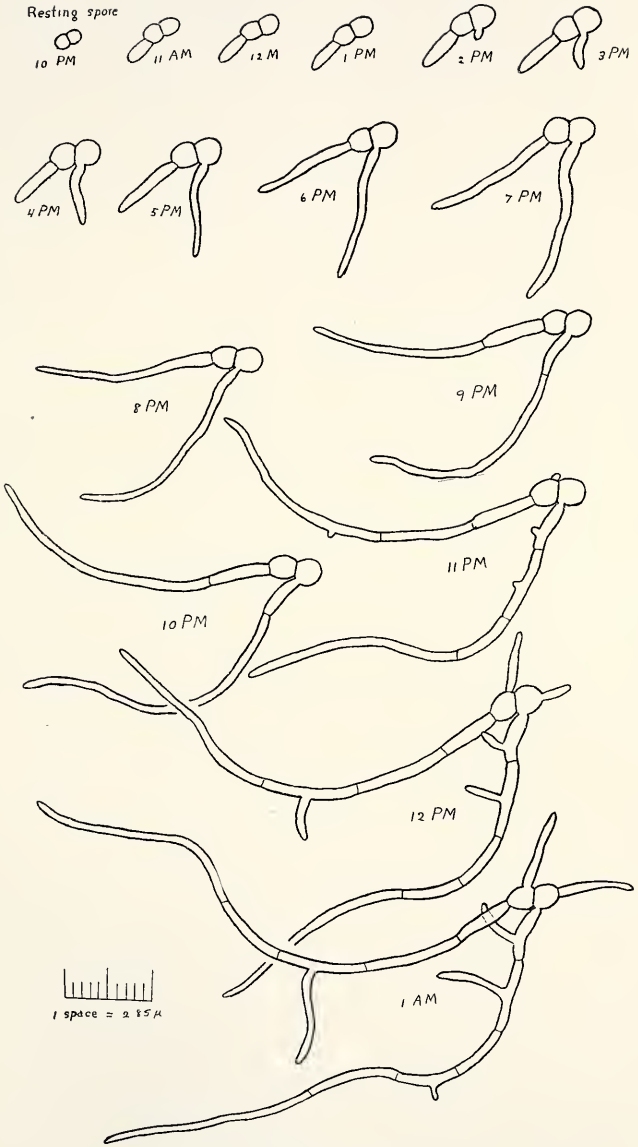

PLATE VII.

Germination of ascospores.

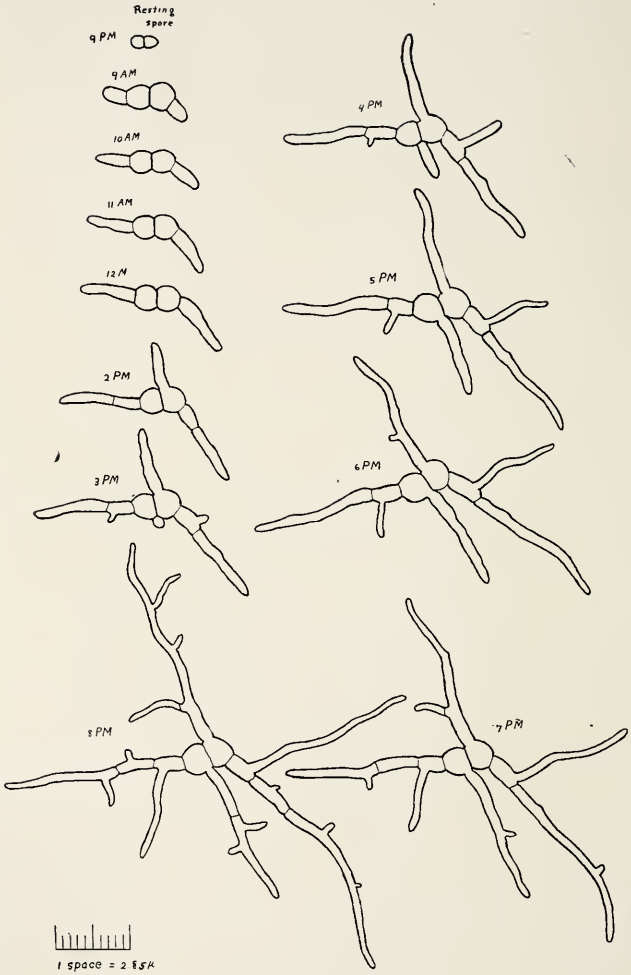

PLATE VIII.

Germination of ascospores.

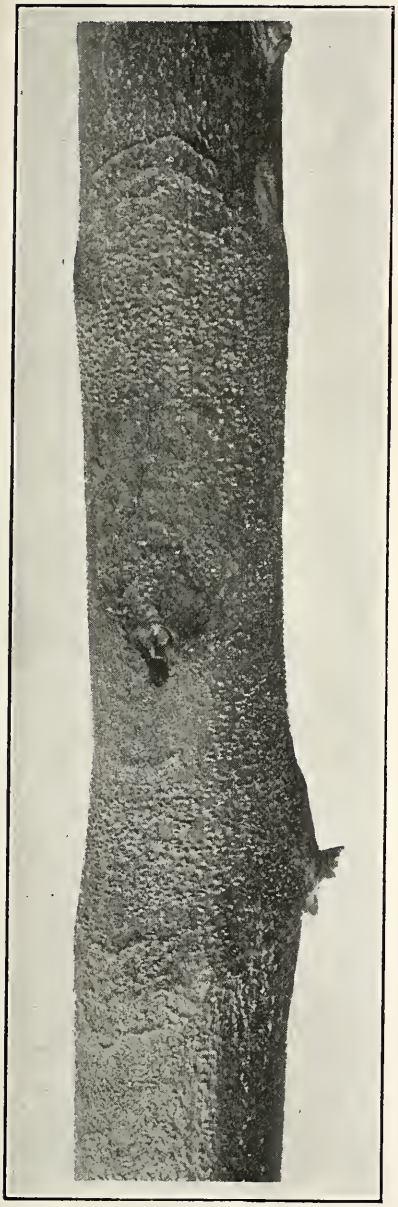

PLATE IX.

Fig. 43.—Canker showing atrophy.

PLATE IX.

Fig. 44.—Canker showing stromata.

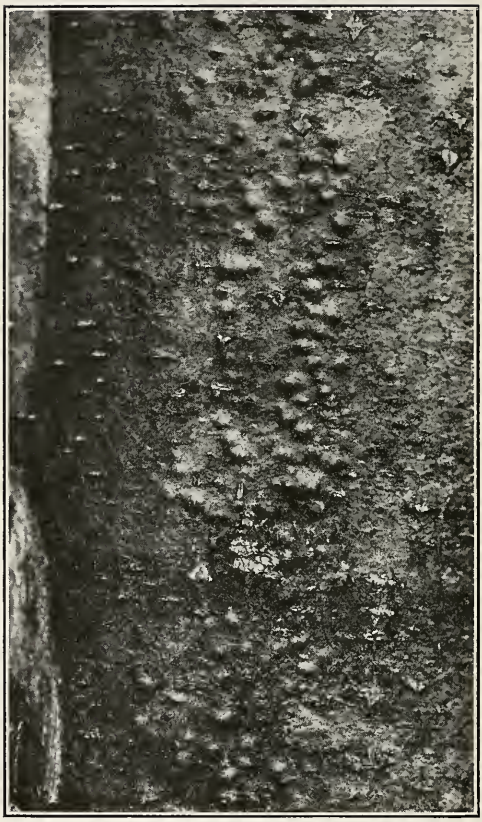

PLATE X.

Fig. 45.—Blister stage of canker.

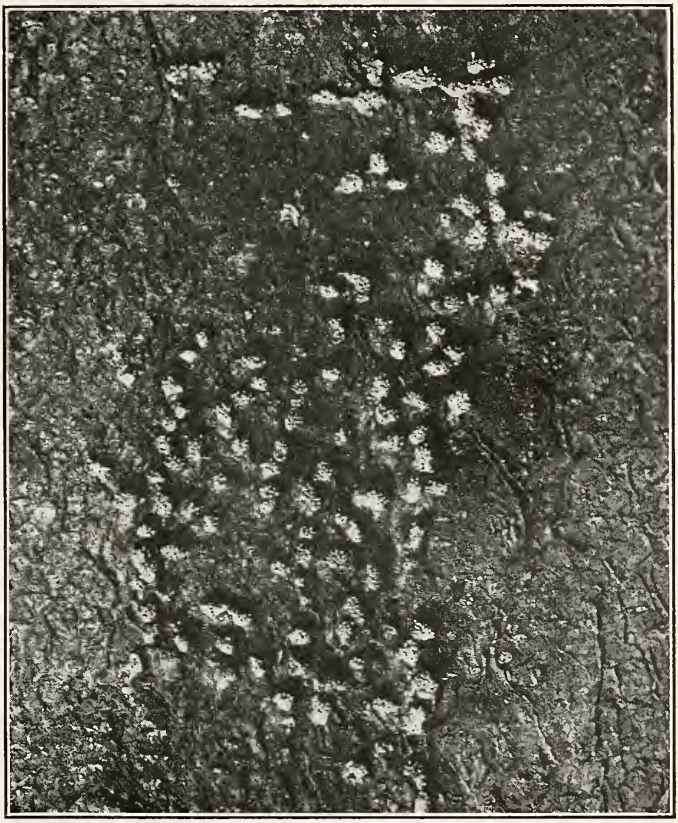

PLATE X.

Fig. 46.—Stromata showing papillae, indicating parithecial stage.

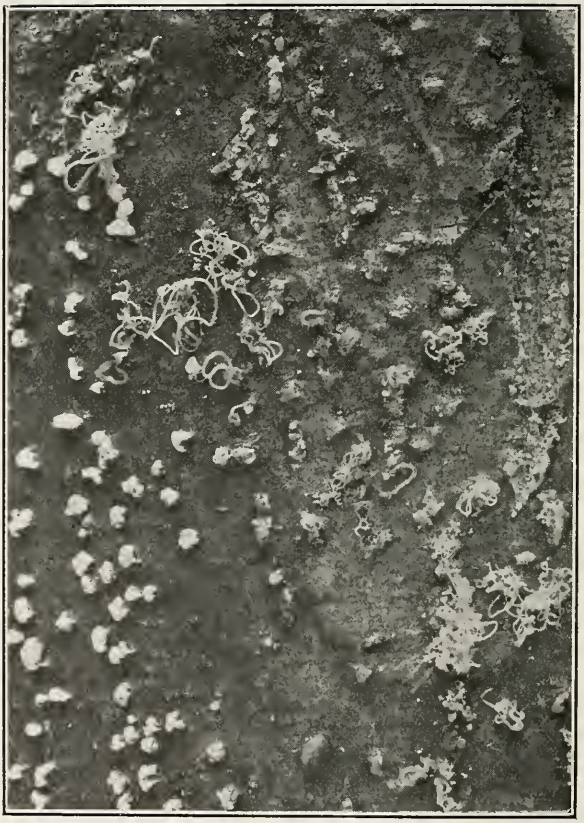

PLATE XI.

Fig. 47.—Spore-horns on smooth bark.

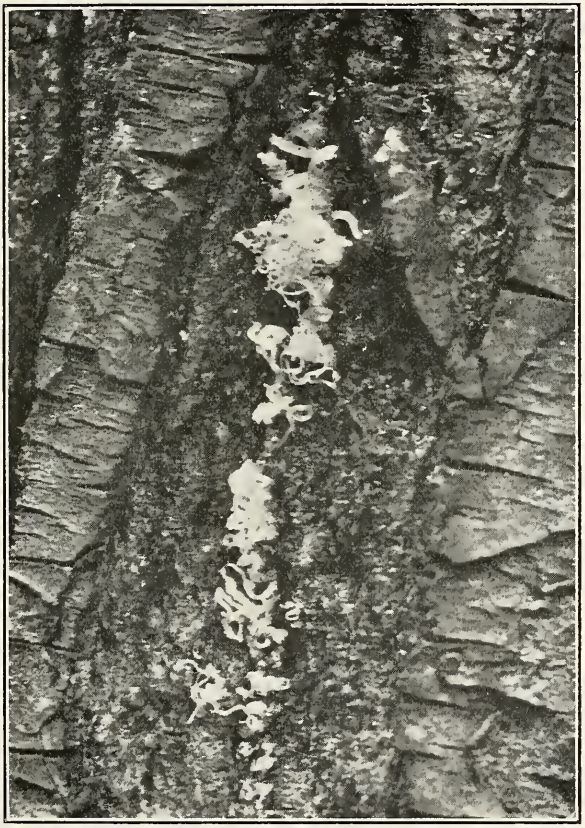

PLATE XI.

Fig. 48.—Sporehorns in crevices of rough bark.

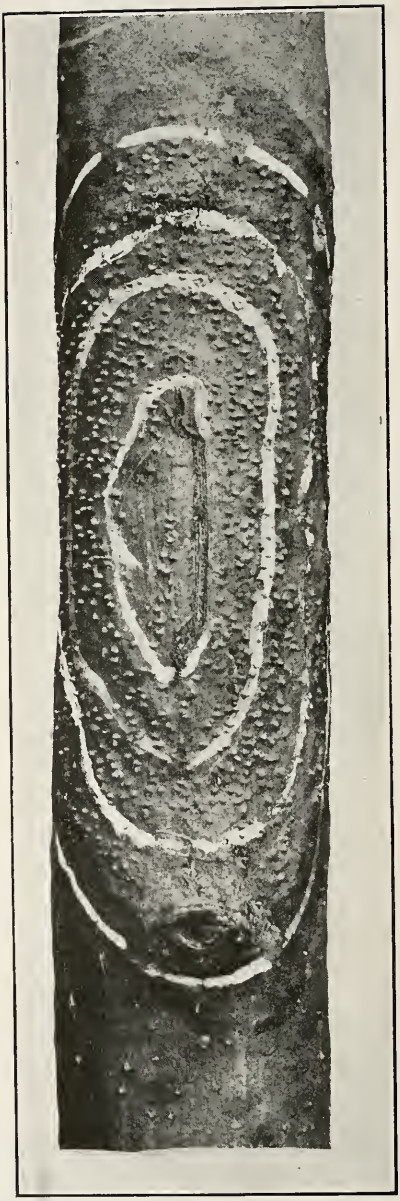

PLATE XII.

Fig. 49.—Outlined canker, indicating monthly growth.

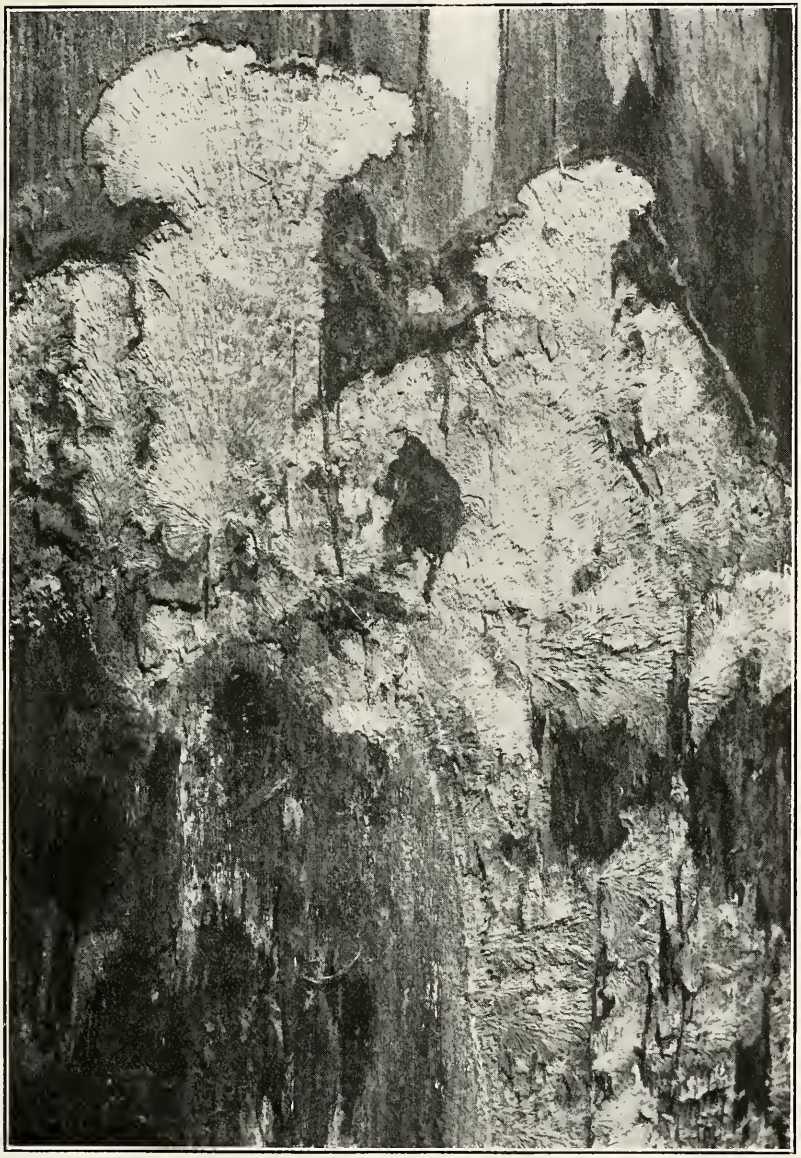

PLATE XIII.

Fig. 50.—Mycelial fans under the chestnut bark.

PLATE XIV.

Fig. 51.—Petri dish culture of pycnidia.

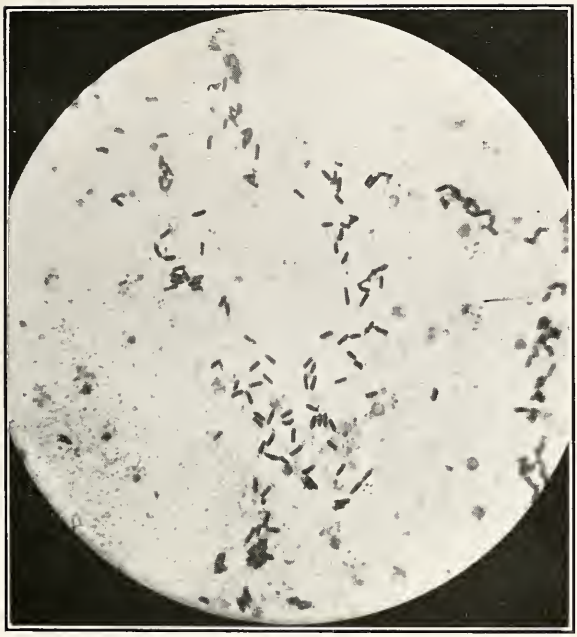

PLATE XV.

Fig. 52.—Photomicrograph of pycnospores.

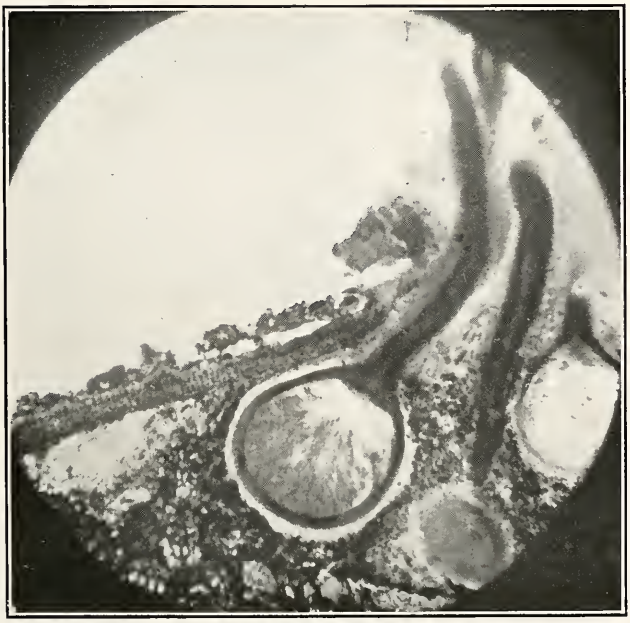

PLATE XV.

Fig. 53.—Vertical section of a perithecium.

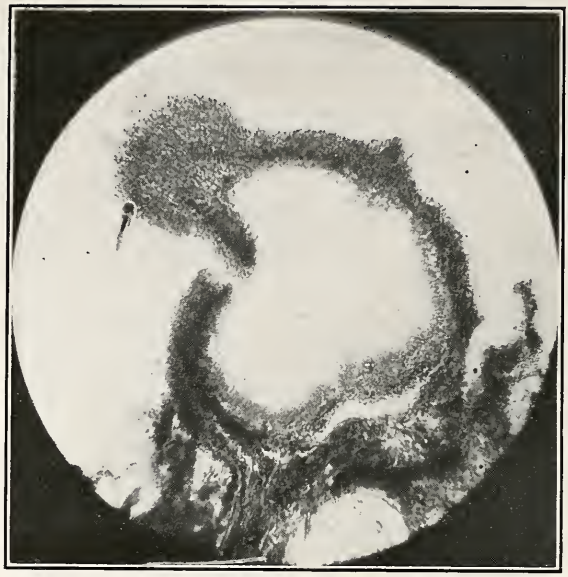

PLATE XVI.

Fig. 54.—Photomicrograph of pycnidium on wood.

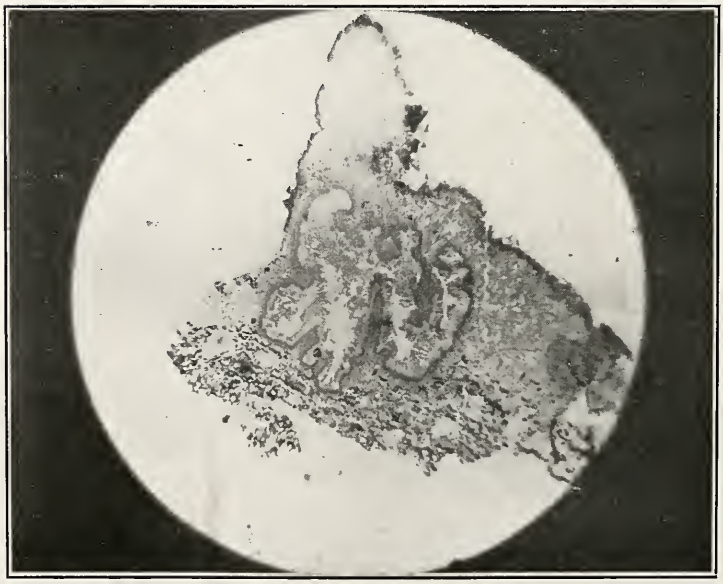

PLATE XVI.

Fig. 55.—Stroma containing labyrinthiform pycnidium.

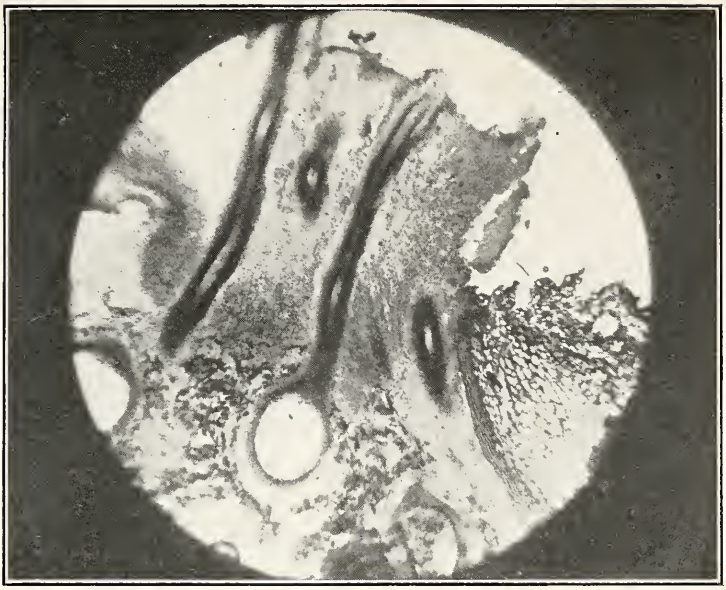

PLATE XVII.

Fig. 56.—Vertical section of stroma showing empty perithecia and black necks.

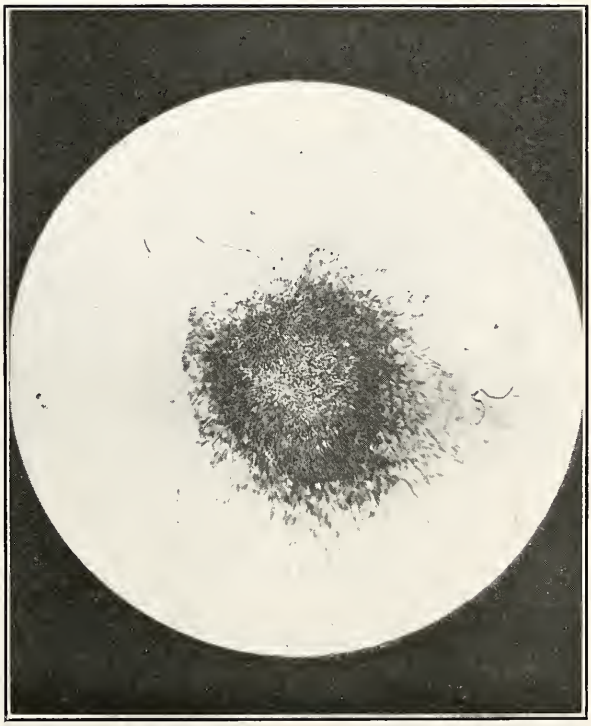

PLATE XVII.

Fig. 57.—Pycnidium on agar showing early stage in the formation of the cavity.

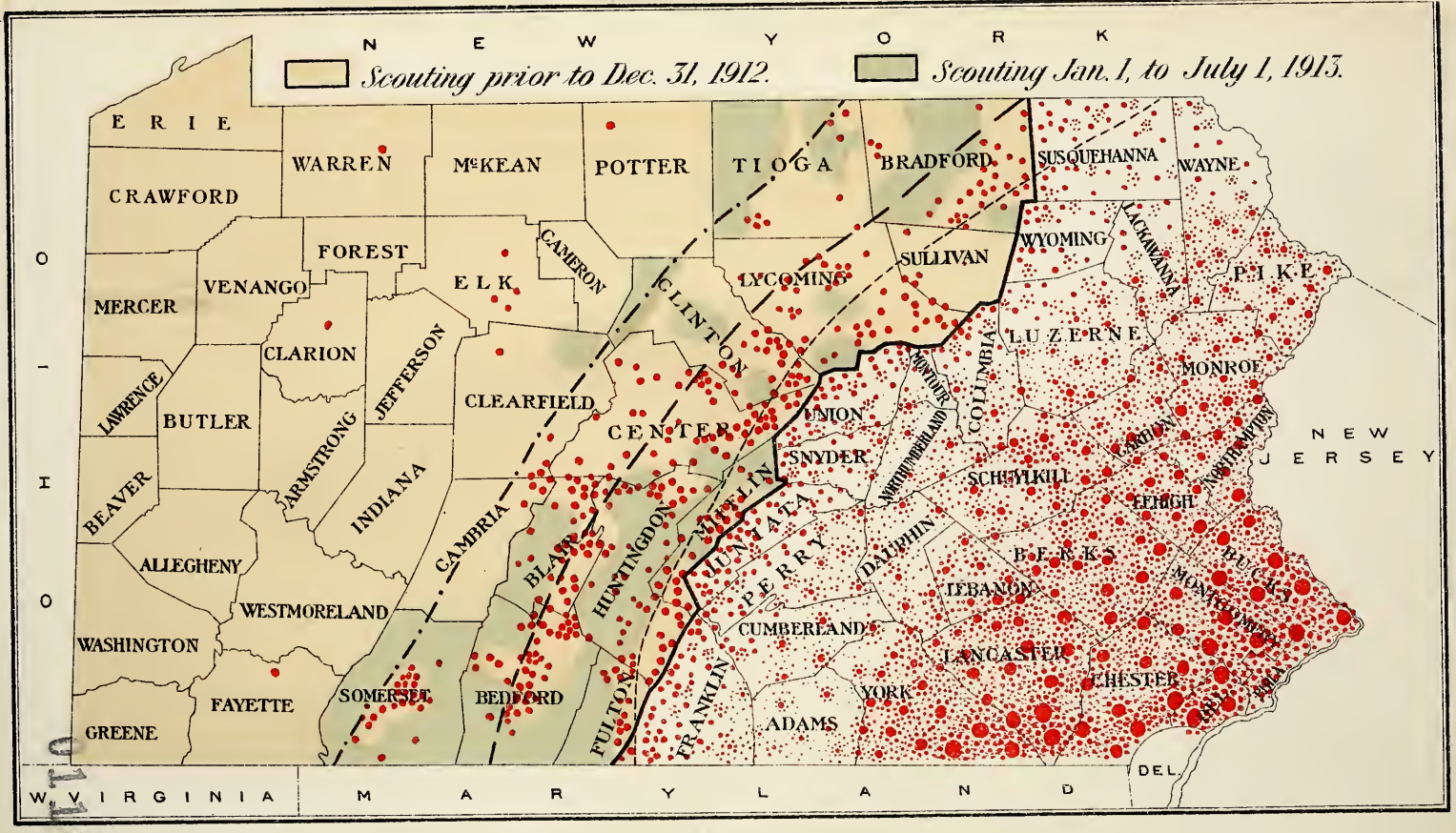

FIG. 1.

SCOUTING AND INFECTION MAP OF PENNSYLVANIA TO JULY 1, 1913.

The dots show the relative progress of the chestnut blight across the State. Each dot in the Western District represents a known spot of infection of from one to one thousand trees. The percentage of blight is shown diagrammatically in the Eastern District. The solid black line is the boundary between the Eastern and Western Districts. The light dotted line is the line of advance infection as determined in 1911. The heavy broken line is the advance [illegible] determined in 1912